

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

APPLICANT: Sivaram Pillarisetti

SERIAL NO.: 10/091,357

GROUP ART UNIT: 1644

FILED: March 1, 2002

EXAMINER: Maher M. Haddad

FOR: Methods and Compositions for the  
Treatment and Prevention of Smooth  
Muscle Cell Proliferation

ATTORNEY DOCKET NO.: 18631 0141 (R18631  
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Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**DECLARATION BY SIVARAM PILLARISETTI, Ph.D.**

Sir:

I, Sivaram Pillarisetti, Ph.D., the undersigned declarant, do hereby declare and state the following:

1. I am the named inventor of the above-identified patent application.
2. I am currently the Vice President of Research, and formerly Director of Research, for Reddy US Therapeutics, Norcross, Georgia, the assignee of the above-identified patent application. **[My resume is attached to this Declaration as EXHIBIT A]**. I earned advanced degrees in biochemistry and molecular biology (Ph.D., Biochemistry/Molecular Biology; M.S. Biochemistry) and I have conducted Postdoctoral Research in molecular biology. From about September 1996 until about July 2000, I was an Assistant Professor at the Department of Medicine, Columbia University, New York, NY and a faculty member at the Long Island Jewish Medical Center of the Albert Einstein College of Medicine, New York, NY. I have both conducted research and directed research programs in the general areas of molecular biology,

lipoproteins, vascular biology, atherosclerosis, angiogenesis and restenosis, cancer, peptide chemistry and biology, cardiovascular inflammation, and inflammatory responses, for at least about the past 15 years. I was an investigator of the American Heart Association (NY affiliate) from 1996-99. I am the author or co-author of over 30 peer-reviewed publications, including publications currently in press, in the areas of endothelial cell and vascular biology, RNA and protein synthesis, molecular biology, and related areas. I am also a co-inventor on 4 issued U.S. patents and 7 published patent applications in the general areas of angiogenesis, restenosis, cellular proliferation, inflammatory responses, vascular and proliferative diseases, glycosidase assays, atherosclerosis, and related arts.

3. I have full knowledge of the subject matter disclosed and claimed in this application.

4. I understand that the U.S. Patent and Trademark Office ("PTO") has asserted that, until the time of *Paka et al.* (abstract Nov. 2, 1999) (herein referred to as "*Paka 1*") and *Paka et al.* (JBC, Dec. 1999, IDS Ref. No. 22) (herein referred to as "*Paka 2*"), apolipoprotein E (apoE) isoforms E2 and E4 were compounds having **unknown** cellular proliferative activity (*i.e.*, not known whether they are i) anti-proliferative, ii) proliferative, or iii) have no effect on cell proliferation). [***Paka 1 & 2 are attached to this Declaration as EXHIBIT B***]. I am co-author and principal investigator on *Paka 1 & 2* and offer the following observations based on my own technical experience and knowledge of the art.

5. *Paka 1* and *Paka 2* propose that apoE stimulates smooth muscle cell production of perlecan, a heparin sulfate proteoglycan, which mediates the antiproliferative activity of apoE. ApoE is a plasma protein whose structural gene locus is polymorphic and includes three major phenotypes (apo E2, E3, and E4) based on three common alleles ( $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$ ). These alleles determine the three homozygous phenotypes E2/2, E3/3, and E4/4 and three heterozygous phenotypes E3/2, E4/2, and E4/3.

As I describe below, at the time of publication of the *Paka* references, the apo E2 and E4 isoforms were compounds having **known** cellular proliferative activity (*i.e.*, shown either to be anti-proliferative, proliferative, or to have no effect on cell proliferation). [**In support of this assertion, *Nathan et al.*, *Yamamoto et al.*, *Cattin et al.*, and *Casscells et al.*, which are discussed below, are attached to this Declaration as EXHIBIT C**].

6. Apo E4's effect on cellular proliferation has been described as early as at least August 25, 1995 as illustrated by the *Nathan et al.* reference, which examines the effects of the apoE isoforms on neurite outgrowth of a murine neuroblastoma cell line. J. Biol. Chem., 270: 19791-19799 (August 25, 1995).

On page 19799, left column, lines 3-4, *Nathan et al.* states, "apoE4 did not effect cell replication, as determined by thymidine incorporation."

And, on page 19793, *Nathan et al.* measures thymidine incorporation into DNA as an indication of cell replication and sees no significant differences in [ $^3\text{H}$ ]thymidine incorporation into DNA with cells incubated with  $\beta$ -VLDL and either apoE3 or apoE4.

Therefore, at least as early as the 1995 publication date of the *Nathan et al.* reference, apoE4's effect on cell replication was known.

7. ApoE2's effect on cellular proliferation has been suggested as early as at least February, 1996 as illustrated by the *Yamamoto et al.* reference. J. American College of Cardiology, Abstracts, Posters, p111A, No. 922-46 (February 1996).

*Yamamoto et al.* reports the incidence of restenosis in patients with different apoE isoforms (*i.e.*, apoE2, apoE3, and apoE4) who underwent percutaneous transluminal coronary angioplasty<sup>1</sup> (PTCA).

According to *Yamamoto et al.*, the apoE4 isoform was related with restenosis (but not the E2 isoform). See page 111A, poster No. 922-46, Table and lines 1-4 of last paragraph.

Therefore, as early as at least the 1996 publication date of the *Yamamoto et al.* reference, apo E2's effect on cell replication was known, *i.e.* the E2 isoform of apoE was shown not to be related with post-angioplasty restenosis, which commonly involves cellular proliferation.

8. Apo E2's effect (as well as E4's effect) on cellular proliferation has also been suggested by *Cattin et al.* *Arteriosclerosis, Thrombosis, and Vascular Biology*, 17: 91-94 (1997).

*Cattin et al.* investigate the possible contribution of apoE allele polymorphism to the carotid intima-media thickness (IMT) (and therefore reflective of cellular proliferation).

According to *Cattin et al.*, "measurements of carotid IMT showed increasing values from E2 to E4 carriers." *Cattin et al.*, Page 91, Abstract lines 13-14.

Therefore, the apo E2 allele's (as well as apo E4's) effect on cell replication was known as illustrated by its effect on IMT, which involves cellular proliferation.

9. In summary, at least *Nathan et al.*, *Yamamoto et al.*, and *Cattin et al.* illustrate that prior to the publication date of the *Paka* references, the apo E2 and E4 isoforms of apoE were compounds having known cellular proliferative activity (*i.e.*, shown either to be anti-proliferative, proliferative, or no effect on cell proliferation).

10. I declare that all statements made herein are believed to be true to the best of my knowledge; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any patent issuing on this application.

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<sup>1</sup> Restenosis, which means the re-occurrence of stenosis, involves cellular proliferation and is common following angioplasty. See, for example, *Casscells et al.*, *Molecular and Cellular Cardiology*, 21: 68-77 (1994).

S. Pillarisetti

Sivaram Pillarisetti, Ph.D.

October 13, 2006

Date



# **Exhibit A**

"EXHIBIT A"

**Sivaram Pillarisetti, Ph.D.**

Reddy US Therapeutics  
3065 Northwoods Circle  
Norcross, GA 30071

**Present position:**

**Vice President of Research**

Reddy US Therapeutics and Dr Reddy Laboratories, Norcross, GA  
Oversee research activities in cardiovascular inflammation programs

**Experience:**

June 2000 – July 2001

**Director of Research**, Reddy US Therapeutics

Dec 98 – July 2000

**Senior Scientist and Director**, Translational Research and therapeutic  
Angiogenesis, North Shore - Long Island Jewish Health System,  
Manhasset, NY

**Assistant Professor** - Albert Einstein College of Medicine, NY

Directed translational research lab focusing on developing peptides and  
peptidomimetics to modulate angiogenesis with therapeutic implications  
in cancer and myocardial and limb ischemia

Sep 96- Jun 99

**Assistant Professor** - Department of Medicine, Columbia University,  
New York, NY 10032

Research in lipoproteins, vascular biology and atherosclerosis

Jan 91- Aug 96

**Staff Associate/Associate Research Scientist** - Department of Medicine  
Columbia University, New York, NY 10032

Research in lipoproteins, vascular biology and atherosclerosis

April 87 - Mar 91

**Post Doctoral Fellow** - Department of Biochemistry, University of  
Connecticut Health Center, Farmington, Connecticut.

Research in molecular biology of eukaryotic protein biosynthesis

**Awards:**

Investigator of the American Heart Association, NYC - 1996-99  
Faculty Research Award, Long Island Jewish Medical Center - 1999  
Atorvastatin Research Award, Parke Davis/Pfizer - 1999

**Teaching:**

Institute of Human Nutrition, Columbia University (1996-98) – Graduate  
program in human nutrition

**Membership:**

American Heart Association, American Diabetes Association and  
American Chemical Society

**Reviewer:**

ATVB, Am J Pathol, Am J Physiol

## Education:

Ph.D.	Biochemistry/Molecular Biology - School of Life Sciences, University of Hyderabad, India.
M.S.	Biochemistry - School of Life Sciences, University of Hyderabad, India.
B.S.	Chemistry (Major) and Biology

## Research Papers:

### Endothelial cells and Vessel wall Biology:

1. S. Pillarisetti, Michael G. Klein and Ira J. Goldberg (1992) Identification of a heparin releasable lipoprotein lipase binding protein from endothelial cells- **J. Biol.Chem.** 267,16517-16522
2. A.Sasaki, S. Pillarisetti, I.J.Goldberg. (1993) Lipoprotein Lipase binding to adipocytes: involvement of heparin sensitive binding sites **Amer.J.Physiol.**-265 (*Endocrinol. Metab.* 28):E 880-88
3. S. Pillarisetti, S.Wadhwani, M.G. Klein, A. Sasaki and I.J. Goldberg (1993) Biotinylation of Lipoprotein lipase and hepatic triglyceride lipase: Application in the assessment of cell binding sites **Anal. Biochem.** 214, 511-516
4. M. Stins, S. Pillarisetti and I.J.Goldberg (1993) Specificity of Lipoprotein Lipase binding to endothelial cells **J.Lipid Res.** 34, 1853-61
5. S. Pillarisetti, S.Y.Choi, L. Curtiss and I.J. Goldberg (1994) An Amino-terminal fragment of Apolipoprotein B binds to Lipoprotein lipase and may facilitate its interaction with Endothelial cells **J. Biol.Chem.** 269, 9409-9413
6. Parthasarathy,N., Goldberg, IJ, S. Pillarisetti, Wagner W. (1994) Identification of a specific Lipoprotein lipase binding oligosaccharide from endothelial heparan sulfate proteoglycans - **J.Biol Chem.** 265, 22391-96
7. S.Y. Choi S. Pillarisetti et al (1995) Lipoprotein lipase binding to lipoproteins involve protein-protein interactions with Apolipoprotein B - **J.Biol.Chem.** 270, 8081-86
8. S. Pillarisetti, JC. Obunike and IJ. Goldberg - Lysolecithin induced alterations of subendothelial heparan sulfate proteoglycans increases monocyte binding to matrix - **J.Biol.Chem.** 270, 29760-29765 (1995)
9. Parthasarathy, N., Goldberg, I.J., S. Pillarisetti, and Wagner, W.D. (1996) Isolation of heparin-derived oligosaccharides containing 2-O-sulfated hexuronic acids by lipoprotein lipase affinity chromatography. **J. Biochem. Biophys. Meth.** 32, 27-32
10. S. Pillarisetti, T. Vanni and I. J. Goldberg (1996) Endothelial cells synthesize and process apolipoprotein B - **J. Biol. Chem.** 271, 15261
11. L. Pang, S. Pillarisetti and I. J. Goldberg. (1996) Cell surface expression of an amino-terminal fragment of apoB increases lipoprotein lipase binding to cells **J. Biol. Chem.** 271, 19518-23
12. JC. Obunike, S. Pillarisetti, L. Paka and I. J. Goldberg (1996) Mechanisms of lipoprotein lipase degradation by adipocytes: LDL receptor related protein and proteoglycan mediated pathways. **J. Lipid Res.** 37, 2439-49
13. S. Pillarisetti, S. Paka, A. Sasaki, B. Yin, N. Parthasarathy, W.D. Wagner, I. Goldberg. (1997) Endothelial heparanase modulation of adipocyte lipoprotein lipase: Evidence that heparan sulfate oligosaccharide functions as an extracellular chaperone. **J.Biol.Chem.** 272, 15753-60 (1997)
14. JC. Obunike, S. Pillarisetti, L. Paka and IJ. Goldberg. (1997) Lipoprotein lipase can function as monocyte adhesion protein - **Arterio. Thromb. Vasc. Biol.** 17, 1414-1420
15. S. Pillarisetti, S. Paka, J. Obunike, L. Berglund, I. Goldberg. (1997) Subendothelial retention of lipoprotein (a). Evidence that reduced heparan sulfate promotes lipoprotein (a) retention by subendothelial matrix **J.Clin.Invest.** 100: 867-874
16. I. Goldberg, L. Pang, S. Paka, W.D. Wagner, and S. Pillarisetti. (1998) Evidence that the amino-terminal region of apolipoprotein B is sufficient for lipoprotein interaction with glycosaminoglycans. **J.Biol.Chem** 273, 35355-35361

17. S. Paka, J. Obunike and S. Pillarisetti. (1999) Apolipoprotein E stimulates endothelial production heparan sulfate rich in biologically active heparin-like domains. A potential mechanism for the anti-atherogenic actions of vascular apoE. **J.Biol.Chem.** 274, 4816-4823
18. Balagopalakrishna, Paka L, Pillarisetti S, Goldberg I. J (1999) Lipolysis-induced iron release from diferric transferrin. Possible role of lipoprotein lipase in ldl oxidation. **J. Lipid Res.** 40, 1347-56
19. S. Paka, Goldberg, I. J., Choi, S. Y. J. Obunike, Saxena, U., Goldberg, I. D. and S. Pillarisetti. Perlecan mediates the anti-proliferative effect of apolipoprotein E on smooth muscle cells: An underlying mechanism for the modulation of smooth muscle cell growth? **J. Biol. Chem.** 274, 36403-36408
20. J. Obunike, S. Pillarisetti et al. (2000) Heparin binding proteins apolipoprotein E and lipoprotein lipase enhance proteoglycan production in cells. **Arterio. Thromb. Vasc. Biol.** 20(1): 111-8
21. Chen G, Paka L, Kako Y, Singhal P, Duan W, Pillarisetti S (2001). A protective role for kidney apolipoprotein E. Regulation of mesangial cell proliferation and matrix expansion. **J Biol Chem.** 276(52): 49142-7.
22. Chen G, Pillarisetti S and Goldberg IJ - Inflammatory cytokines and fatty acids regulate endothelial cell heparanase expression – revised for **Biochemistry**
23. Kako, Y et al Inhibition of Atherosclerosis in Severely Hyperlipidemic Mice by Gene Therapy with an Endothelial Anti-apoptotic Factor –in review

#### RNA and Protein synthesis:

24. S. Pillarisetti, Gary Vellekamp and Murray Deutscher - A role for lipids in the functional and structural properties of rat liver aminoacyl-tRNA synthetase complex - **J. Biol.Chem.** 263,18891-96 (1988)
25. S. Pillarisetti and R. Mayasundari - Qualitative and quantitative variation of tRNA in certain invertebrates - **J.Biosci.**14,153-162 (1989)
26. S. Pillarisetti and Murray P. Deutscher - Free fatty acids associated with high molecular weight aminoacyl-tRNA synthetase complex influence its structure and function - **J.Biol.Chem.** 265,5774-5779 (1990).
27. S. Pillarisetti and Murray P.Deutscher - Existence of two forms of rat liver arginyl-tRNA synthetase suggests channeling of aminocyl-tRNA for Protein Synthesis **Proc.Natl.Acad.Sci.USA** - 87,3665-3669 (1990)

#### Reviews:

24. S. Pillarisetti - Lipoproteins and vessel wall - Implications of new research on our understanding of Atherosclerosis - **New developments in Cardiology and Cardiac surgery.**(Pub. Escorts Heart Institute) p1-11 (1993)
25. S. Pillarisetti - Detecting and isolating ligands interacting with lipoprotein lipase - In **Mammalian Lipases and Phospholipases.** (Eds. M.Doolittle and K.Reue; Methods in Mol. Biology series, J. Walker ed.,) 1999
26. S. Pillarisetti – Lipoprotein modulation of subendothelial heparan sulfate proteoglycans and atherogenicity – **Trends Card. Vasc. Med.** 10(2): 60-5 2000
27. Saxena, U. and S. Pillarisetti (2000) Endothelial Cell Sites for Drug Regulation- New Discoveries in endothelium and lipoprotein Metabolism (in press)
28. Saxena U and Pillarisetti, S – New approaches for treatment of diabetic nephropathy: the endothelium as a target for drug discovery. **Expert Opin Ther Targets.** 2001 Oct; 5(5): 539-545.
29. Pillarisetti, S and Saxena U - Lipoprotein lipase as a therapeutic target for dyslipidemia. **Front Biosci.** 2003 Jan 1; 8: d238-41.
30. Pillarisetti, S Alexander C and Saxena U – Atherosclerosis – novel targets and therapeutics –**Curr Med Chem Cardiovasc Hematol Agents.** 2004 Oct;2(4):327-34
31. Pillarisetti, S and Saxena U – Role of oxidative stress and inflammation in the origin of diabetes. **Expert Opin Ther Targets.** 2004 (in press)

**Patents:****Issued:**

1. Pillarisetti S. and Goldberg IJ - US6156315 Method for inhibiting the binding of low density lipoprotein to blood vessel matrix 2000-12-05  
Application in atherosclerosis
2. Pillarisetti S. and Goldberg ID - US6610726 Compositions and agents for modulating cellular proliferation and angiogenesis 2003-08-26  
Application in restenosis/angiogenesis
3. Pillarisetti S. and Goldberg ID - US6589997 Small-molecule modulators of hepatocyte growth factor/scatter factor activities 2003-07-08  
Identified small molecule agonists and antagonists of growth factors for application in angiogenesis and cancer
4. Pillarisetti S. Wang D and Saxena U. US6656699 Methods and compositions for glycosidase assays 2003-12-02

**Published:**

5. Pillarisetti et al - US20030036103A1 Methods and compositions for diagnosis and treatment of vascular conditions 2003-02-20
6. Pillarisetti S. and Goldberg ID - US20030022924A1 Compositions and agents for modulating cellular proliferation and angiogenesis 2003-01-30
7. Pillarisetti S - US20020182587A1 Methods and compositions for the treatment and prevention of smooth muscle cell proliferation 2002-12-05
8. Pillarisetti S. Cahoon S and Saxena U. US20020086282A1 Methods and compositions for detecting compounds that modulate inflammatory responses 2002-07-04
9. US20020077293A1 Methods and compositions for the treatment of inflammatory diseases 2002-06-20
10. Pillarisetti, S and Saxena U - WO03065008A2 Methods and compositions for identification and therapeutic use of genes involved in vascular and proliferative diseases 2003-08-07
11. Timmer, R, Alexander, A, Pillarisetti, S, Saxena U and Campbell K - WO03024926A3 Methods and compositions of novel triazine compounds 2003-03-27

# **Exhibit B**



404 870-4842

vessels demonstrated significantly reduced neointima at 21 days in vessels treated with the pro-pro-eccepin A gene compared with neointimal area in those given a control gene ( $p < 0.05$ ). **Conclusion:** Thus, the needle injection catheter appears to be useful for local intravascular gene delivery. *In vivo* gene transfer of cecopins may be of therapeutic relevance in restenosis prevention by limiting cell proliferation.

2886

### Perlecan, Heparan Sulfate Proteoglycan, Mediates the Anti-Proliferative Effect of Apolipoprotein E: An Underlying Mechanism for the Modulation of Smooth Muscle Cell Growth?

Latha Paka, North Shore - Long Island Jewish Health System, Manhasset, NY; Joseph C. Obunike, Columbia Univ, New York, NY; Sungshin Y. Choi, Palo Alto Med Ctr, Palo Alto, CA; Sivaram Pillarsetti, North Shore - Long Island Jewish Health System, Manhasset, NY

We recently showed that apoE stimulates endothelial production of heparan sulfate (HS) enriched in heparin-like sequences. Since heparin and HS are potent inhibitors of smooth muscle cell (SMC) proliferation, in this study we determined whether the anti-proliferative effect of apoE is due to increased HS production. In confluent SMC, apoE (5  $\mu$ g/ml) increased  $^3$ S- $^35$ SO $_4$  incorporation into cells by 24% and media by 36%. The increase in the medium was exclusively due to an increase in HS (2.1 fold) and apoE did not alter chondroitin and dermatan sulfate proteoglycans (PG). In proliferating SMC, apoE inhibited bFGF/EGF stimulated  $^3$ H-thymidine incorporation into DNA by 50%; however, despite decreasing cell number, apoE increased the ratio of  $^3$ SO $_4$  to  $^3$ H-thymidine from 2 to 3.5 suggesting increased HS per cell. Purified HSPG from apoE-stimulated cells inhibited cell proliferation in the absence of apoE. ApoE did not inhibit proliferation of endothelial cells which are relatively resistant to heparin-inhibition. Analysis of the conditioned medium from apoE stimulated cells revealed that the HSPG increase was in perlecan and apoE also stimulated perlecan mRNA expression by  $>2$  fold. The ability of apoE isoforms to inhibit SMC proliferation correlated with their ability to stimulate perlecan production and E2 and E4 were less effective in stimulating perlecan. These data suggest that the anti-proliferative effect of apoE is due to its ability to increase perlecan HSPG, a potent inhibitor of SMC proliferation. Because other growth modulators also regulate perlecan expression, we postulate that this is a key pathway in the regulation of SMC growth.

2887

### Endochondral Calcification Is Responsible for Coronary Artery Calcification

Lorraine A. Fitzpatrick, R. Turner, K. Shogren, E. Ritman, Mayo Clin & Fdn, Rochester, MN

Calcification is a highly regulated process involving specific matrix proteins responsible for mineralization, growth factors, cartilage as a substrate, and collagen and crystal deposition. Ovariectomy enhances proliferative chondrocytes in skeletal tissue and increases coronary artery calcification. We developed a model of coronary calcification in normal Sprague-Dawley aged rats that permits ready access to calcified coronary arteries. Hearts from oophorectomized (OVX) aged rats and intact (sham-operated) rats were scanned by X-ray micro-CT to determine quantitative, high-resolution, 3-dimensional architecture. Large amounts of calcium were present in the coronary arteries of hearts from OVX rats. Hydroxyapatite was confirmed histologically with special staining; none was present in the coronary arteries from intact animals. Histological analysis revealed a large focus of cartilage in the OVX heart, consistent with endochondral ossification. Endochondral ossification begins in cartilage formed from mesenchymal cells. These hypertrophic chondrocytes form the primary center of ossification, which then degenerates and is invaded by vascular endothelial cells. Type II collagen, a chemotactic factor for chondrocytes and a marker for the cartilage phenotype was prominent throughout the matrix. These data reveal, for the first time, that cartilage is a precursor tissue in this model of coronary artery calcification. This implicates new methods of intervention to prevent coronary calcification targeted at cartilage proteins and gene products.

## Arteriosclerosis, Thrombosis & Vascular Biology, High Blood Pressure Research, Basic Cardiovascular Sciences, Cardiopulmonary & Critical Care, Kidney in Cardiovascular Disease, Stroke:

Interaction Between the Renin-Angiotensin System and Endothelial Cells

Tuesday Afternoon

Georgia World Congress Center Exhibit Hall

Abstracts 2888-2898

2888

### Human Endothelial Cells Bind and Activate Prorenin

Mark MED van den Eijnden, Jasper J. Saris, Wim Sluiter, Maarten ADH Schalekamp, Frans HM Derkx, Alexander HJ Danser, Erasmus Univ, Rotterdam Netherlands

Vascular angiotensin generation depends on uptake of (pro)renin from the circulation. In support of this concept, we have recently demonstrated that human umbilical vein endothelial

cells (HUVECs) bind and internalize prorenin (PR) via a mannose 6-phosphate (M6P) receptor-dependent mechanism (Admiraal et al., 1999). It was the aim of the present study to investigate the binding kinetics and to assess the capacity of HUVECs to form renin by proteolytic cleavage of the prosegment. HUVECs were cultured in a chemically defined medium with recombinant human PR (range 0.05-20 nM;  $n=4$ ) at 4°C or 37°C for 4 hrs, or loaded with 2 nM PR ( $n=3$ ) for 2 hrs at 4°C, washed, and then incubated at 37°C. Non-M6P receptor-dependent binding was determined by adding 10 mM M6P to the medium, and by incubating cells with M6P-free PR, obtained with a M6P receptor affinity column (Faust et al., 1987). Intact PR and activated PR were measured in cell lysates by enzyme-kinetic assay (4°C experiments) or immunoradiometric assays (37°C experiments), with monoclonal antibodies directed against the prosegment of PR, or a renin-specific epitope, respectively. Internalization of PR was confirmed by acid-wash. Results:  $K_d$  and  $B_{max}$  were  $2.5 \pm 0.8$  nM and  $665 \pm 60$  sites/cell. At 37°C, the amount of PR internalized via M6P receptors was more than 15 times  $B_{max}$ . Once inside the cells, intact PR decreased with a  $t_{1/2}$  of  $2 \pm 1$  hrs. This was due to the formation of renin, which reached a maximum after 2 hrs. PR not containing the M6P signal was not bound by HUVECs. **Conclusions:** HUVECs bind PR to surface M6P receptors with high affinity. 2) PR internalization is greatly enhanced by receptor recycling, and 3) HUVECs contain an enzyme that converts PR to renin. These processes may contribute to local angiotensin generation in the vascular wall.

2889

### Evidence for the Functional Importance of ACE-Dependent *In-Situ* Angiotensin II Generation in the Human Forearm

Alexander HJ Danser, Erasmus Univ, Rotterdam Netherlands; Marjan A van Dijk, Ingrid Kroon, Peter C Chang, Leiden Univ Med Ctr, Leiden Netherlands; Maarten ADH Schalekamp, Erasmus Univ, Rotterdam Netherlands

To assess the importance, for vasoconstriction, of *in-situ* angiotensin (Ang) II generation, as opposed to Ang II delivery via the circulation, we determined forearm vasoconstriction in response to Ang I (0.1-10 ng/kg/min) and Ang II (0.1-5 ng/kg/min) in 16 normotensive male volunteers (age 18-67 yr). Changes in forearm blood flow (FBF) were registered with venous occlusion plethysmography. Arterial and venous blood was sampled under steady-state conditions to quantify forearm fractional Ang I-II conversion. Ang I and II exerted the same maximal effect ( $71 \pm 4\%$  (mean  $\pm$  SEM) and  $75 \pm 4\%$  decrease in FBF, respectively) with similar potencies ( $EC_{50}$  4.8 (0.9-17.4) nM (mean (range)) for Ang I and 2.1 (0.7-6.7) nM for Ang II). Forearm fractional Ang I-II conversion was 22 (13-40)%. Concomitant infusion of the ACE inhibitor enalaprilate (80 ng/kg/min) inhibited the effects of Ang I and reduced fractional conversion to 1 (0.1-8)%, thereby excluding a role for Ang I-II converting enzymes other than ACE (e.g. chymase). Concomitant infusion of the AT $_1$  receptor antagonist losartan (3 mg/kg/min) inhibited the effects of Ang II. **Conclusion:** The similar potencies of Ang I and II in the forearm, combined with the fact that 25% of arterially delivered Ang I is converted to Ang II suggest that *in-situ* generated Ang II is more important for vasoconstriction than circulating Ang I. Local Ang II generation in the forearm depends on ACE and results in vasoconstriction via binding to AT $_1$  receptors.

2890

### Angiotensin II-Receptor-Antagonism Improves Endothelium-Mediated Vasodilation in Patients with Coronary Artery Disease: Role of Nitric Oxide and Bradykinin

Christoph Kohler, Burkhard Hornig, Daniel Schlink, Helmut Drexler, Hannover Med Sch, Hannover Germany

Impaired flow-dependent, endothelium-mediated vasodilation (FDD) is an early finding in patients with coronary artery disease (CAD). In contrast to ACE-inhibition, angiotensin-II type 1-receptor-antagonists (AT $_1$ ) do not affect bradykinin degradation and their effect on FDD is unknown. We therefore evaluated the acute effects of the AT $_1$  candesartan (Cand; 800  $\mu$ g/min i.v., 5 min) on FDD of the radial artery in 20 patients with CAD (no previous ACE-inhibition or AT $_1$ ). To investigate the role of nitric oxide or bradykinin for the effects of AT $_1$ , we determined the effect of the bradykinin-B2-receptor antagonist HOE140 (90 mg/min, 5 min; group A,  $n=10$ ) or of the NO-synthase inhibitor L-NMMA (7 mmol/min, 5 min; group B,  $n=10$ ) in co-infusion with Cand on flow-dependent vasodilation. Arterial diameter was measured by high-resolution ultrasound (10 MHz; precision 2  $\mu$ m) at rest and after 8 minutes of wrist occlusion. Diameter changes (D%), representing FDD, were determined at control, after Cand, after co-infusion of Cand and L-NMMA or HOE140 respectively (all data: mean  $\pm$  SEM; \*  $p < 0.05$  vs. control; \*  $p < 0.05$  vs. Cand). **Conclusion:** The AT $_1$  candesartan improves FDD in patients with CAD. This effect is abolished after co-infusion with L-NMMA suggesting a NO-mediated mechanism. The effect of Cand on FDD is severely reduced after coinfusion with HOE140, supporting the concept that both, bradykinin/the B $_2$ -receptor and nitric oxide are involved in AT $_1$ -mediated increase in FDD.

	control	Cand	Cand + NMMA	Cand + HOE
D% (grp.A)	7.7 $\pm$ 0.3	10.8 $\pm$ 0.3*	-	5.4 $\pm$ 0.3*
D% (grp.B)	7.4 $\pm$ 0.4	9.9 $\pm$ 0.4*	4.2 $\pm$ 0.2*	-



# Perlecan Mediates the Antiproliferative Effect of Apolipoprotein E on Smooth Muscle Cells

AN UNDERLYING MECHANISM FOR THE MODULATION OF SMOOTH MUSCLE CELL GROWTH?\*

(Received for publication, July 23, 1999, and in revised form, August 19, 1999)

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Apolipoprotein E (apoE) is known to inhibit cell proliferation; however, the mechanism of this inhibition is not clear. We recently showed that apoE stimulates endothelial production of heparan sulfate (HS) enriched in heparin-like sequences. Because heparin and HS are potent inhibitors of smooth muscle cell (SMC) proliferation, in this study we determined apoE effects on SMC HS production and cell growth. In confluent SMCs, apoE (10 µg/ml) increased <sup>35</sup>SO<sub>4</sub> incorporation into PG in media by 25–30%. The increase in the medium was exclusively due to an increase in HSPGs (2.2-fold), and apoE did not alter chondroitin and dermatan sulfate proteoglycans. In proliferating SMCs, apoE inhibited [<sup>3</sup>H]thymidine incorporation into DNA by 50%; however, despite decreasing cell number, apoE increased the ratio of <sup>35</sup>SO<sub>4</sub> to [<sup>3</sup>H]thymidine from 2 to 3.6, suggesting increased HS per cell. Purified HSPGs from apoE-stimulated cells inhibited cell proliferation in the absence of apoE. ApoE did not inhibit proliferation of endothelial cells, which are resistant to heparin inhibition. Analysis of the conditioned medium from apoE-stimulated cells revealed that the HSPG increase was in perlecan and that apoE also stimulated perlecan mRNA expression by >2-fold. The ability of apoE isoforms to inhibit cell proliferation correlated with their ability to stimulate perlecan expression. An anti-perlecan antibody completely abrogated the antiproliferative effect of apoE. Thus, these data show that perlecan is a potent inhibitor of SMC proliferation and is required to mediate the antiproliferative effect of apoE. Because other growth modulators also regulate perlecan expression, this may be a key pathway in the regulation of SMC growth.

Apolipoprotein E (apoE)<sup>1</sup> is a key ligand for several lipoprotein receptors and plays a major role in the hepatic clearance of

remnant lipoproteins (1, 2). In recent years, however, several nontraditional functions of apoE have emerged that are related either to its antiatherogenic function or its role in Alzheimer's disease (3–9). For example, expression of apoE in the vessel decreased atherosclerosis in apoE-null mice without significant changes in plasma lipoproteins (4, 5). Recently, Fazio *et al.* (5), by transplanting apoE null macrophages into normal C57BL/6 mice, increased atherosclerosis without altering lipoprotein profile. How apoE protects the vessel wall from atherogenesis is not clear. Possible antiatherogenic roles of vascular apoE include promotion of reverse cholesterol transport (6), inhibition of lipoprotein oxidation (7), inhibition of lipase-mediated low density lipoprotein retention (8), inhibition of platelet aggregation (9), inhibition of smooth muscle cell (SMC) proliferation (10), and, as we recently showed, increasing endothelial heparan sulfate (HS) (11).

Heparin and HS are biologically active glycosaminoglycans (GAG) composed of alternating residues of uronic acid (glucuronic acid in HS and iduronic acid in heparin) and glucosamine (12). HSPGs have several vasoprotective effects; the best characterized among these is their ability to inhibit SMC proliferation (13–15). Although the antiproliferative effect of apoE has been realized for many years (16–18, 10), how apoE inhibits cell proliferation is not known. Our previous studies showed that apoE increased HSPG production in endothelial cells (11). Thus, it raises the possibility that apoE-mediated inhibition of SMC proliferation may be due to its ability to induce HS production in cells. In the present study, we show that apoE stimulates SMC production of perlecan HSPGs, which mediates the antiproliferative activity of apoE.

## MATERIALS AND METHODS

Heparinase I and heparitinase (heparinase III) and chondroitin ABC lyase were purchased from Seikagaku America Inc. (Bethesda, MD). Aqueous solutions of [<sup>35</sup>S]sulfate were from Amersham Pharmacia Biotech. [<sup>3</sup>H]Leucine and [<sup>3</sup>H]glucosamine were from NEN Life Science Products. ApoE3 was either purchased from Calbiochem (La Jolla, CA) or purified from conditioned medium of Chinese hamster ovary cells transfected with apoE cDNA (19) by heparin-agarose chromatography. ApoE2 and apoE4 isoforms were from Calbiochem. Perlecan antibody was from Zymed Laboratories Inc. (South San Francisco, CA).

**Cells**—Rat and human aortic SMCs were kindly provided by Dr. L. Rabbani (Department of Medicine, Columbia University) (20). Data with rat SMCs are presented below. Initial experiments were also performed with human SMCs, and similar results were obtained. SMCs were grown in basal medium supplemented with growth factors, basic fibroblast growth factor and epidermal growth factor (Clonetics, San

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<sup>1</sup> The abbreviations used are: apoE, apolipoprotein E; HS, heparan sulfate; PG, proteoglycan; HSPG, heparan sulfate proteoglycan; SMC,

smooth muscle cell; GAG, glycosaminoglycans; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

Diego, CA). Bovine aortic endothelial cells were isolated and cultured as described (21). The cells (5–15 passages) were grown in minimal essential medium containing 10% fetal bovine serum (Life Technologies, Inc.).

**Metabolic Labeling**—PGs were radiolabeled with either [ $^{35}\text{S}$ ]sulfate or [ $^3\text{H}$ ]leucine for the indicated time periods. Medium PGs were collected and purified by DEAE-cellulose chromatography (see below). Cell associated PGs were assessed by extracting cells with 50 mM Tris buffer, pH 7.4, containing 4 M urea, 1% Triton X-100, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. To study the effects of apoE, confluent SMCs were incubated in culture medium containing  $^{35}\text{SO}_4$  and the indicated concentrations of apoE for 24 h. Cell and medium PG levels were assessed.

**DEAE Cellulose Chromatography of PGs**—To determine changes in PGs, DEAE-cellulose chromatography was performed as described previously (21, 22). A DEAE-cellulose column was equilibrated with 50 mM Tris buffer, pH 7.4, containing 4 M urea, 0.1 M NaCl, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% CHAPS. The column was washed with the same buffer and with buffer containing 0.25 M NaCl, and PGs were eluted with the same buffer containing 0.5 M NaCl. Fractions containing radioactivity ( $^{35}\text{SO}_4$ ) were pooled and dialyzed against minimal essential medium overnight and counted. To determine the relative proportion of HSPGs and chondroitin and dermatan sulfate PGs, an aliquot of the pooled fraction was incubated in 50 mM sodium acetate buffer, pH 5.2, with 1 unit/ml each of heparinase and heparitinase or with 0.5 units of chondroitin ABC lyase for 16 h at 37 °C. The reaction mixture was precipitated either with 0.5 volumes of 1% cetylpyridinium chloride or with 3 volumes of ethanol to precipitate undigested GAG. Radioactivity in the supernatant and pellet was determined.

**SMC Proliferation**—To determine the effects of apoE or HS on SMC proliferation, cells were plated at low density ( $8 \times 10^4$ /well) and cultured for 24–48 h in the presence or absence of apoE or HSPGs. Cell number was counted with hemacytometer, and net growth was determined (15). Alternatively, SMCs were cultured in the above conditions; cells were then labeled with [ $^3\text{H}$ ]thymidine for 6 h, and radioactivity incorporated into the DNA was determined by trichloroacetic acid precipitation of the cell lysate.

**Determination of Perlecan Protein and mRNA**—To determine changes in perlecan protein, control and apoE-treated (10  $\mu\text{g}/\text{ml}$ ) SMCs were labeled with [ $^3\text{H}$ ]leucine for 24 h (steady state). PGs were isolated from SMC medium and purified by DEAE-cellulose chromatography. Purified PGs were immunoprecipitated with an anti-perlecan antibody (100-fold diluted), and immunoprecipitates were analyzed by 5% SDS-PAGE. Perlecan (molecular mass, >550 kDa) was identified by autoradiography.

For Northern blotting, a 497-base pair polymerase chain reaction product representing domain I of perlecan (forward and reverse primers with sequences 5'-GCTGAGGGCCTACGATGG-3' and 5'-TGCCAGGCGTCGGAAC-3', respectively) was generated by reverse transcription-polymerase chain reaction of endothelial cell RNA. Northern blotting of total RNA from control and apoE-treated (5  $\mu\text{g}/\text{ml}$  for 24 h) SMCs was performed using  $^{32}\text{P}$ -labeled perlecan probe. RNA load was normalized by determining 18 S RNA.

**Data Analysis**—Results are expressed as mean  $\pm$  S.D. Experiments were done in triplicate and repeated at least once. Statistical analyses were performed by Student's *t* test to determine the significance of change. A significance difference was considered for *p* values equal to or less than 0.05.

## RESULTS

**ApoE Increases Sulfate Incorporation into SMC HSPGs**—Previous studies showed that addition of apoE increased HSPG production in endothelial cells but not in macrophages, which predominantly synthesize chondroitin and dermatan sulfate PGs (15). Similarly, HSPGs represent only ~25% of total PGs synthesized by SMCs. To determine whether apoE increased HSPGs in SMCs, confluent monolayers of SMCs were incubated with apoE (10  $\mu\text{g}/\text{ml}$ ) for 16 h, and PGs in cellular and secreted pools were determined following DEAE-cellulose purification. In different experiments, the total PG level in cells was increased by 15–22% and in medium by 25–30% in apoE-treated cells (Fig. 1A). Because HSPGs in the media can act as inhibitors of cell proliferation, we determined HSPGs in control and apoE media. The heparinase-sensitive radioactivity, rep-

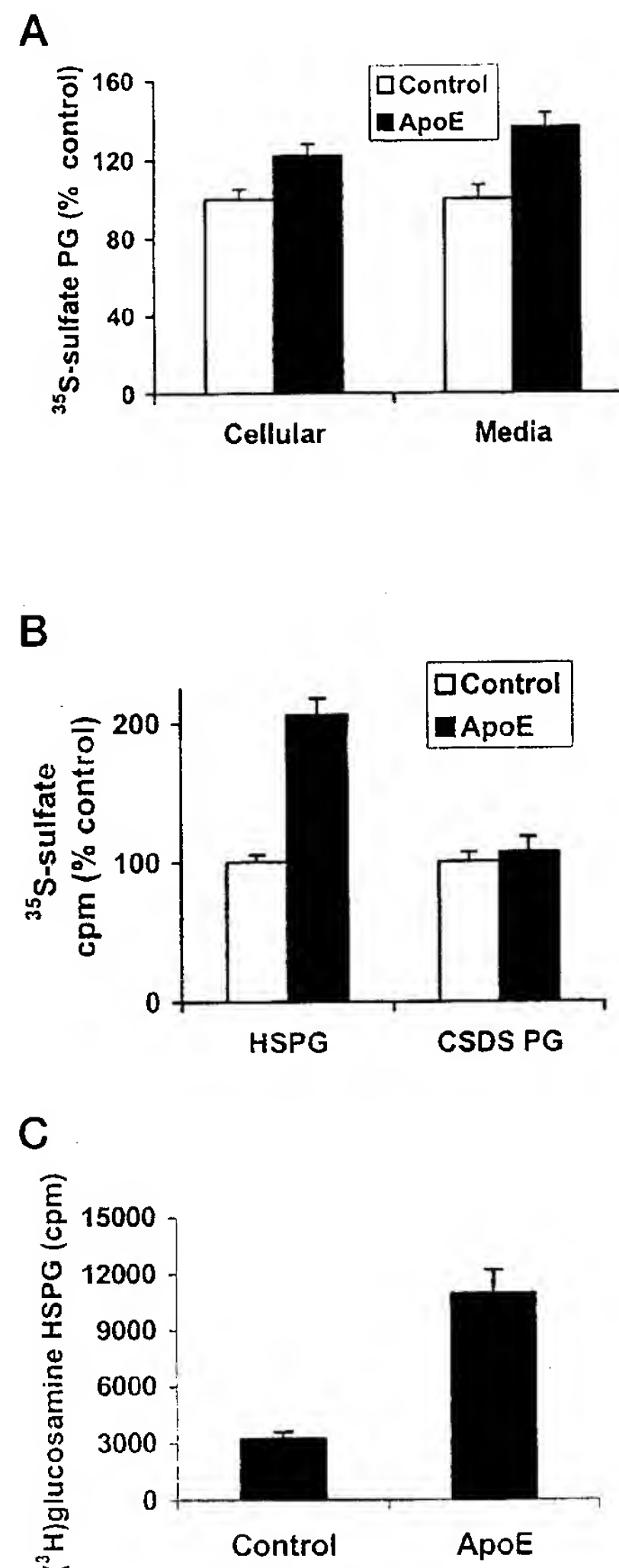
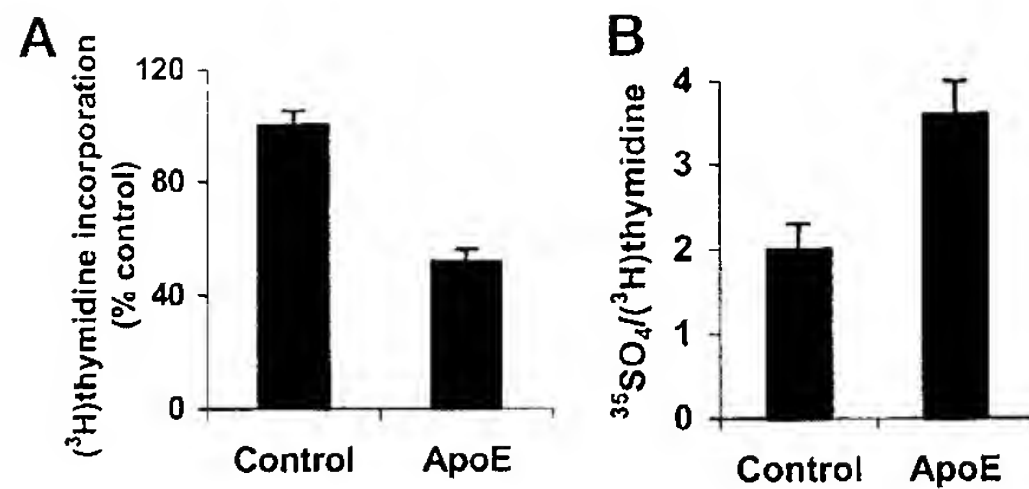


FIG. 1. A, apoE stimulates SMC PGs. Confluent monolayers of SMCs in 24-well plates were incubated with apoE (10  $\mu\text{g}/\text{ml}$ ) in growth medium (basal medium containing 5% serum and growth factors) containing [ $^{35}\text{S}$ ]sulfate (50  $\mu\text{Ci}/\text{well}$ ) for 24 h under culture conditions. Sulfate-labeled PGs in the cells and media were determined after purification by DEAE-cellulose chromatography. Values represent mean  $\pm$  S.D. B, apoE increases SMC medium HSPGs. Purified  $^{35}\text{S}$ -PGs from control and apoE-treated SMC media were treated with 1 unit/ml each of heparinase and heparitinase for 6 h at 37 °C and precipitated with cetylpyridinium chloride.  $^{35}\text{S}$ -Radioactivity in precipitate and supernatant were determined. The digested GAG in the supernatant represent HSPGs and the undigested GAG in the precipitate represent chondroitin and dermatan sulfate PGs. C, apoE stimulates [ $^3\text{H}$ ]glucosamine incorporation into SMC medium HSPGs. To determine whether apoE increased HS GAG, PGs were labeled as in A, but with [ $^3\text{H}$ ]glucosamine and incorporation into medium HSPGs was determined. Values represent mean  $\pm$  S.D. Compared with control, apoE increased [ $^3\text{H}$ ]glucosamine incorporation into PGs by 3-fold, suggesting increased HS GAG. However, compared with data in B, the increase in HS GAG was higher than the increase in sulfation, suggesting that medium HSPGs are relatively under-sulfated in apoE-treated cells.

resenting HSPGs, was increased by 108% in apoE-treated cells (Fig. 1B). The amounts of chondroitin and dermatan sulfate PGs, which constitute ~75% of total medium PGs, were not altered by apoE-treatment. These data show that apoE treatment of SMCs results in an increase specifically in HSPGs. As in endothelial cells (15), this increase was found to be primarily due to an increase in synthesis (not shown).





**FIG. 2. ApoE decreases SMC proliferation (A) but increases HSPGs per cell (B).** Subconfluent SMCs (~35–40%) were incubated with control medium containing  $^{35}\text{SO}_4$  (50  $\mu\text{Ci}/\text{ml}$ ) or medium containing apoE (10  $\mu\text{g}/\text{ml}$ ) and  $^{35}\text{SO}_4$  for 24 h.  $^3\text{H}$ Thymidine was then added and incubated for 6 h. Medium was collected, PGs were purified by DEAE-cellulose chromatography, and  $^{35}\text{SO}_4$  radioactivity in PGs was determined. Cells were washed, and  $^3\text{H}$ thymidine incorporation into DNA was assessed. Data in A show that apoE inhibited proliferation ( $^3\text{H}$ thymidine incorporation) by ~49%. Despite decreasing cell number, apoE increased the ratio of  $^{35}\text{SO}_4$  to  $^3\text{H}$ thymidine (B), suggesting increased HSPG production per cell.

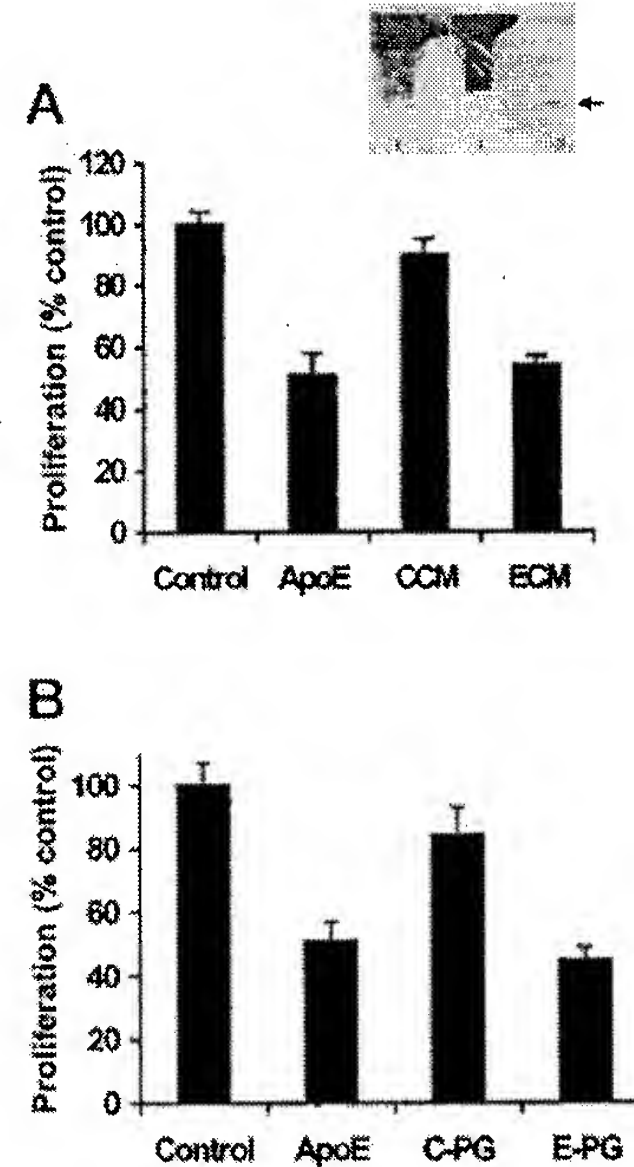
We also determined the effect of apoE on  $^3\text{H}$ glucosamine incorporation into PGs. ApoE increased  $^3\text{H}$ glucosamine incorporation into HSPGs by about 3.5-fold (Fig. 1C). Thus, the ratio of  $^3\text{H}$ glucosamine to  $^{35}\text{SO}_4$  in HSPGs was increased approximately by 1.75, suggesting that although HS GAG were increased by apoE, these HS are relatively under-sulfated.

**ApoE Inhibits SMC Proliferation**—The above experiments were done on confluent SMCs. We next determined apoE effects on proliferating SMCs. Previous studies showed that apoE inhibits SMC proliferation stimulated by serum or platelet-derived growth factor (10). The growth medium in the current experiments contained serum, basic fibroblast growth factor, and epidermal growth factor. In different experiments, the addition of apoE to the medium inhibited cell proliferation by 45–55% (both cell number and  $^3\text{H}$ thymidine incorporation; Fig. 2A) in 24 h. This inhibition was greater than that previously observed with 25  $\mu\text{g}$  of apoE (10).

We examined whether apoE altered HSPGs in proliferating cells. Because the cell number is decreased by apoE, by comparing the ratios of  $^{35}\text{SO}_4$  to  $^3\text{H}$ thymidine, we determined the amount of PGs per cell in control and apoE-treated cells (Fig. 2B). Despite decreasing the cell number, apoE increased the ratio of  $^{35}\text{SO}_4$  to  $^3\text{H}$ thymidine (from 2 to 3.46), suggesting increased HSPGs per cell.

**HSPGs from ApoE-treated Cells Are Potent Antiproliferatives**—We first examined whether apoE-treated cells contained antiproliferative substances in the medium. Conditioned medium was collected from control and apoE-treated SMCs, and apoE was removed by immunoprecipitation (Fig. 3, inset). These media were then added to subconfluent SMCs, and cell growth was determined after 24 h (Fig. 3A). Control conditioned medium (CCM) inhibited SMC growth by 18% compared with control medium. Conditioned medium from apoE-treated cells (ECM) inhibited cell proliferation by 51%, suggesting that apoE treatment stimulated the production of antiproliferative substances.

We next determined whether HSPGs in apoE-conditioned medium mediated inhibition of cell proliferation. PGs from control and apoE-conditioned media were purified by DEAE-cellulose chromatography. This procedure also removed any remains of apoE from the medium (not shown). Equal amounts ( $^{35}\text{SO}_4$  cpm) of purified PGs were then added to subconfluent SMCs in growth medium, and cell growth was determined. PGs from apoE-treated cells (Fig. 3B, E-PG) inhibited SMC proliferation to an extent similar to that of apoE. PGs from control cells (Fig. 3B, C-PG), although at a similar level, inhibited SMC



**FIG. 3. ApoE-treated SMC media contain antiproliferative substances.** Confluent SMCs were incubated with control medium or medium containing apoE for 24 h, and conditioned medium was collected. Conditioned media collected from control and apoE-treated cells were incubated with a polyclonal apoE antibody followed by precipitation with protein A-Sepharose. Supernatants of conditioned media were tested for antiproliferative effect. Subconfluent SMCs were incubated for 24 h in growth medium alone (Control), growth medium containing apoE (ApoE), or conditioned medium from control cells (CCM) or apoE-treated cells (ECM).  $^3\text{H}$ Thymidine incorporation was determined. Values represent mean  $\pm$  S.D. Conditioned medium from apoE-stimulated cells (ECM) inhibited cell proliferation better than conditioned medium from control cells (CCM), similar to the effect of apoE. *Inset*, SDS-PAGE and Coomassie staining of ECM to demonstrate that apoE is removed from ECM by immunoprecipitation: One-milliliter aliquots of ECM were either concentrated by Centricon-10 filtration (lane 1) or subjected to immunoprecipitation with apoE antibody. Following immunoprecipitation, ECM supernatant was concentrated by Centricon-10 filtration (lane 2). Immunoprecipitate (lane 3) contains a single band of molecular weight 34,000 (arrow), which was present in the ECM (lane 1) but not in the supernatant following immunoprecipitation (lane 2). B, PGs from apoE-treated SMCs are antiproliferative.  $^{35}\text{SO}_4$ -PGs from conditioned media of control (C-PG) and apoE-treated cells (E-PG) were purified by DEAE-cellulose chromatography. Equal amounts of  $^{35}\text{S}$ -labeled PGs were then added to fresh subconfluent SMCs and incubated for 24 h.  $^3\text{H}$ Thymidine incorporation was determined for 6 h. PGs from apoE-treated cells (E-PG) but not from control cells (C-PG) inhibited cell proliferation similar to that of apoE.

proliferation by only 18%. These data suggest that HSPGs from apoE-treated SMCs are antiproliferative.

**ApoE Inhibits SMC Proliferation Stimulated by Lysolecithin**—We previously showed that lysolecithin and oxidized low density lipoprotein treatment decreases extracellular HSPGs (23, 24), and others have shown that these agents stimulate SMC proliferation (10, 25). We therefore determined whether apoE ability to increase HSPGs would block lysolecithin effects on SMC proliferation. Incubation of SMC with lysolecithin decreased  $^{35}\text{SO}_4$  incorporation into PGs by 36% (not shown). Concomitant with this decrease, lysolecithin increased  $^3\text{H}$ thymidine incorporation into DNA (Fig. 4). Lysolecithin effects on SMC proliferation were completely abolished in the presence of apoE. These data suggest that agents that modulate HSPGs influence cell proliferation.

**ApoE Increases Perlecan Production in SMCs**—We next characterized the antiproliferative HSPGs in apoE-stimulated cells. Perlecan is the major HSPG secreted by vascular cells (26). To determine whether apoE increased perlecan secretion,

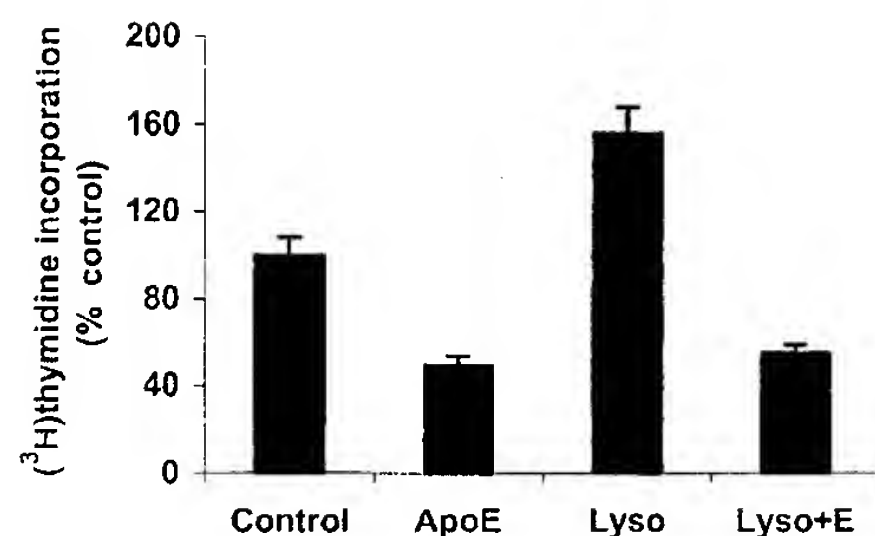


FIG. 4. **ApoE inhibits lysolecithin-induced SMC proliferation.** Subconfluent SMCs were incubated with medium containing 25  $\mu$ M lysolecithin (*Lyso*), apoE (10  $\mu$ g/ml), or lysolecithin + apoE (*Lyso+E*) and incubated for 24 h. Proliferation was determined by [<sup>3</sup>H]thymidine incorporation. Values represent mean  $\pm$  S.D.

DEAE-cellulose-purified, [<sup>3</sup>H]leucine-labeled (core protein-labeled) HSPGs from control and apoE-treated cells were immunoprecipitated by anti-perlecan antibody and analyzed by SDS-PAGE and autoradiography (Fig. 5). The radioactivity associated with a protein of  $M_r$  ~550,000 (perlecan has a core protein of ~400,000 containing three HS chains of  $M_r$  ~50,000–70,000) was increased by apoE. Concomitant with protein increase, apoE also increased perlecan mRNA by greater than 2-fold. These data suggest that the antiproliferative HSPG in SMC medium is perlecan.

**Effects of ApoE Isoforms**—We next studied the effects of apoE isoforms to determine whether antiproliferative activity correlated with increase in perlecan HSPGs (Fig. 6). ApoE3, the most common isoform of apoE, showed maximum stimulation on perlecan production and inhibition on cell proliferation (45%). ApoE2 and apoE4 did not significantly increase perlecan HSPGs or inhibit cell proliferation. These data further show that the antiproliferative effect of apoE correlates with its ability to stimulate perlecan HSPGs.

**The Antiproliferative Effect of ApoE Requires Perlecan**—We next determined whether the antiproliferative effect of apoE is mediated by perlecan. Subconfluent SMCs were incubated with control medium or apoE medium containing nonspecific antibody or anti-perlecan antibody (Fig. 7A). Perlecan antibody did not affect cell growth under control conditions. ApoE inhibited [<sup>3</sup>H]thymidine incorporation into DNA approximately by 48%. In the presence of perlecan antibody, this inhibition was reduced to about 9%. We performed the same experiment with human vascular SMCs (Fig. 7B). ApoE inhibited SMC proliferation by 71%. Perlecan antibody, however, under control conditions stimulated SMC proliferation by 30–35%. The effect of apoE was completely reversed by perlecan antibody. These data suggest that perlecan mediates the antiproliferative effect of apoE both in rat and human SMCs.

#### DISCUSSION

HSPGs are thought to be important for blood vessel homeostasis, blood clotting, atherogenesis, and atherosclerosis. Atherosclerotic vessels have reduced HSPGs, and previous studies have shown that apoE-HDL increased endothelial HS, which in turn could decrease the occurrence of events related to atherosclerosis (11). ApoE was able to increase secretion of HSPGs in both endothelial cells (11) and SMCs (present study). Because subendothelial matrix HSPGs produced by apoE-treated endothelial cells showed strong inhibition of SMC growth, we postulated that actions of vascular apoE would regulate SMC proliferation in the subendothelial space (11). Our current data show direct effects of apoE on SMC HSPGs and thus identify a mechanism for the known antiatherogenic effect of apoE.

The present data strongly suggest that the antiproliferative effect of apoE is due to induction of perlecan HSPGs in SMCs.

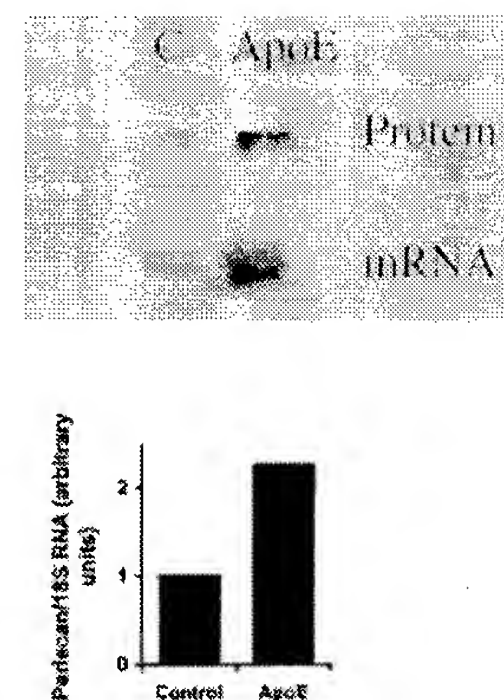


FIG. 5. **ApoE increases perlecan protein and mRNA.** SDS-PAGE of perlecan. Control (C) and apoE-treated (10  $\mu$ g/ml) SMCs were labeled with [<sup>3</sup>H]leucine for 24 h. PGs were isolated from SMC medium and purified by DEAE-cellulose chromatography. Purified PGs were immunoprecipitated with anti-perlecan antibody and analyzed by 5% SDS-PAGE and autoradiography. A single band with a molecular mass slightly higher than 550 kDa (apolipoprotein B (molecular mass, ~550 kDa) was used as a marker) was observed, the intensity of which was increased in apoE-treated SMCs. No other bands were seen on SDS gels. ApoE increases perlecan mRNA. A 497-base pair polymerase chain reaction product representing domain I of perlecan was used for Northern blotting. Total RNA was isolated from control and apoE-treated (10  $\mu$ g/ml, 24 h) SMCs, and Northern blotting was performed using <sup>32</sup>P-labeled perlecan probe. A single band with a  $M_r$  of ~15 kb was observed. Densitometric analysis (bars) showed that perlecan band intensity, expressed as a ratio of perlecan to 18 S RNA, was increased by apoE by more than 2-fold.

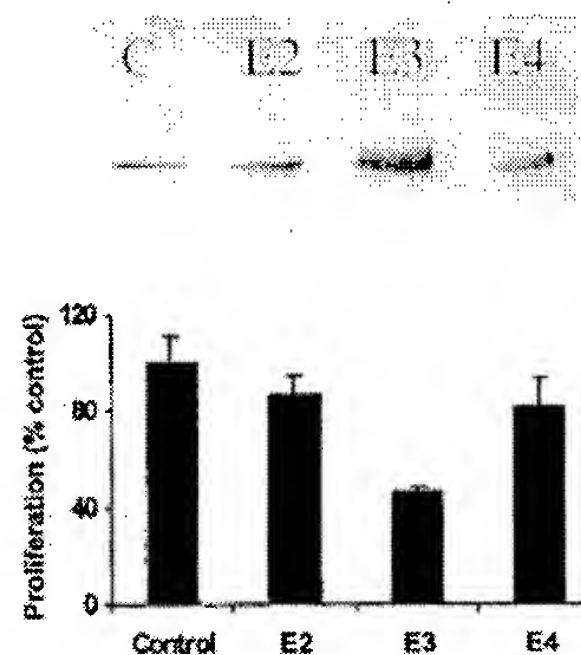


FIG. 6. **Effects of apoE isoforms on perlecan production and proliferation.** Subconfluent SMCs were incubated with medium alone or medium containing 10  $\mu$ g/ml of apoE isoforms (E2, E3, and E4) for 24 h. [<sup>3</sup>H]Thymidine incorporation was determined. In another experiment, confluent SMCs were incubated with apoE isoforms in medium containing <sup>35</sup>SO<sub>4</sub>, and incorporation into medium PGs was determined as described in Fig. 1.

1) HSPGs isolated from apoE-stimulated cells inhibited proliferation better than those from control SMCs. 2) ApoE countered the effects of lysolecithin, which is known to decrease extracellular HSPGs. 3) The antiproliferative effect of apoE isoforms correlated with their ability to stimulate perlecan HSPGs. 4) An anti-perlecan antibody blocked the inhibitory effect of apoE on SMC growth, showing that perlecan is required for the antiproliferative effect of apoE. Moreover, apoE did not inhibit proliferation of endothelial cells, which are not sensitive to HSPG inhibition (27) ([<sup>3</sup>H]thymidine: control, 4780  $\pm$  270 cpm; apoE, 5540  $\pm$  330 cpm). Our data are also consistent with the observation that both apoE (28) and HSPGs (29) inhibit mitogen-activated protein kinase, a key signaling pathway in cell growth.

ApoE treatment increased both perlecan mRNA and protein. Perlecan, the major HSPG of endothelial cells and SMCs (26), consists of a core protein of  $M_r$  ~450,000 to which three HS



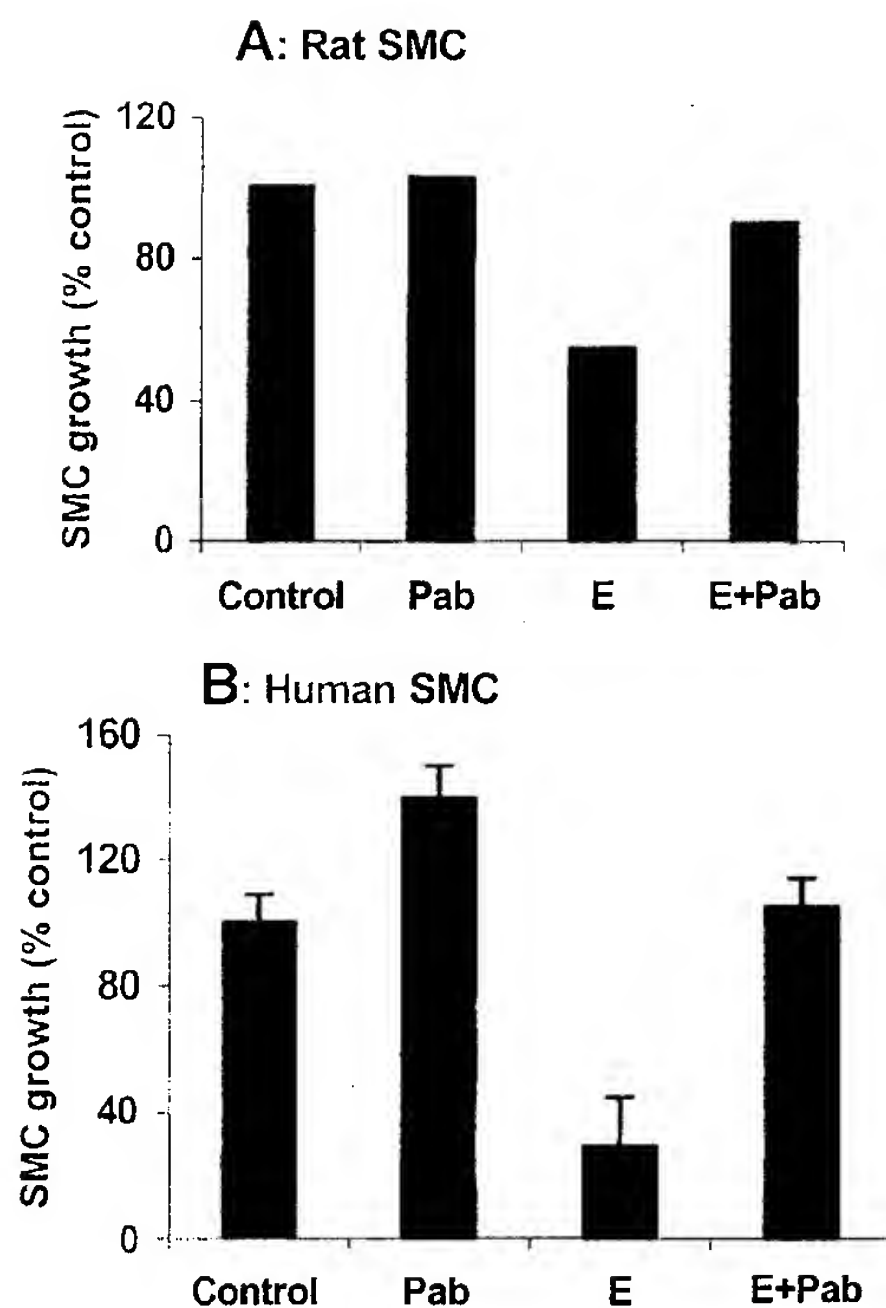


FIG. 7. The antiproliferative effect of apoE requires perlecan. Subconfluent rat SMCs (A) or human SMCs (B) were incubated with medium alone (control), medium containing 10  $\mu$ g/ml perlecan antibody (Pab), medium containing 10  $\mu$ g/ml apoE (E), or apoE and perlecan antibody (E+Pab) for 24 h. [ $^3$ H]Thymidine incorporation was determined.

chains with a molecular mass of  $\sim 70$  kDa are attached at one end of the molecule. The core protein consists of five consecutive domains with homologies to molecules involved in control of cell proliferation, lipoprotein uptake, and cell adhesion. Perlecan core protein can mediate cell adhesion and interact with other matrix proteins, and it plays a critical role in matrix assembly. HS chains of perlecan can bind growth factors. Although *in vitro* all isolated HSPGs are effective inhibitors of SMC proliferation, the identity of the antiproliferative HSPGs *in vivo* is not known. Cell surface HSPGs are required for the mitogenic activity of several growth factors (12) and thus are unlikely to inhibit cell growth. Extracellular HSPGs, on the other hand, either by sequestering the growth factors or by other signaling mechanisms, can inhibit cell proliferation. Our data for the first time identify direct effect of perlecan on SMC growth and its requirement to mediate the antiproliferative effect of apoE.

Perlecan was shown to negatively correlate with SMC proliferation (30), and it was shown to inhibit Oct-1, a growth-related transcription factor (31). In certain cell types, however, blocking perlecan production via antisense DNA inhibited cell growth (32–34). It is conceivable that perlecan under normal conditions is required for matrix assembly and cell growth; however, excess perlecan in medium that is not deposited into the matrix may block growth factor binding and activity. Support for this comes from the observations that perlecan core protein can bind cell surface integrins and support cell growth (35) and that serum induces perlecan production at early time periods but decreases at later time periods (36).

It is not clear why excess perlecan remained in the medium. It is conceivable that the amount of perlecan in the matrix is saturating, leading to accumulation in the medium. Alternatively, perlecan produced by apoE-stimulated cells is different. The data shown in Fig. 1C suggest that HS chains in perlecan

are under-sulfated. Perlecan interaction with surrounding matrix proteins, such as laminin and collagen, requires both core protein and HS chains (37). It is conceivable that reduced sulfation affects perlecan HS interactions with laminin or other perlecan molecules, thereby reducing its ability to incorporate into matrix.

The antiproliferative effect of perlecan is likely due to the HS chains. Although it is not entirely clear how HS inhibits cell proliferation, several mechanisms have been proposed (29, 38–40). We are surprised, however, by the observation that perlecan antibody, which reacts with domain III of perlecan, completely blocked perlecan effect. Domain III is thought to mediate cell adhesion (26), and attachment to the matrix and spreading is a key part of cell growth. Perlecan antibody, as shown in Fig. 7 in control conditions, either did not affect or stimulated SMC growth. However, when added during the seeding of SMCs, perlecan antibody inhibited SMC growth by  $>40\%$  (not shown). We propose that perlecan in matrix is required for cell growth and that excess unincorporated perlecan may engage the SMC surface molecules involved in cell attachment and spreading. Studies have shown both adhesive and antiadhesive functions for perlecan (26, 41, 42), and the current studies may offer an explanation why this occurs.

Vascular cells produce a variety of growth promoters and inhibitors (43). Physiologically relevant agents that stimulate SMC proliferation include platelet-derived growth factor, thrombin, oxidized low density lipoprotein, and lysolecithin. Vascular cell derived growth inhibitors include transforming growth factor- $\beta$ , nitric oxide/cGMP, and apoE. Going through published literature on perlecan regulation, we identified an interesting possibility that perlecan may be the key for modulation of SMC growth. Platelet-derived growth factor (44), thrombin (45), serum (36), oxidized low density lipoprotein, and lysolecithin (23, 24), which stimulate SMC growth, decrease perlecan. In contrast, the antiproliferative agents transforming growth factor- $\beta$  (46), apoE (present study), and even heparin (47) stimulate perlecan expression. Thus, we postulate that modulation of perlecan is key to regulation of cell growth.

The observation that apoE up-regulates perlecan may have implications in other physiological and pathological processes. Perlecan is known to modulate angiogenesis (48). It remains to be determined whether apoE could be an angiogenic factor *in vivo*. ApoE induction of perlecan may also have implications in the pathogenesis of Alzheimer's disease. Brains of patients with Alzheimer's disease accumulate deposits of  $\beta$ -amyloid protein. The  $\beta$ -amyloid protein-containing deposits in the vessel wall are primarily associated with SMCs, endothelial cells, and the surrounding matrix, and studies showed that perlecan is associated with the  $\beta$ -amyloid protein deposits (49). It remains to be determined whether apoE can induce perlecan production in neuronal cells, and if this occurs, it is conceivable that production of soluble perlecan may compete for  $\beta$ -amyloid protein binding to matrix perlecan. Soluble HS-like molecules can inhibit amyloid progression in mice (50).

How apoE stimulates perlecan and what cell surface molecule(s) mediates apoE actions remain to be determined. Based on previous studies, both HSPGs and receptor-associated protein-sensitive pathways may mediate apoE effects on perlecan (11). ApoE- $\beta$ -very low density lipoprotein, which binds poorly to HSPGs (51), does not inhibit DNA synthesis (52), and RAP at high concentrations could affect apoE binding to HSPGs (53); thus, it is conceivable that cell surface HSPGs directly mediate apoE effect (54). It should be noted that demonstration of requirement for cell surface HSPGs in mediating the antiproliferative effect of apoE is difficult as agents that interfere with cell surface HSPGs, such as heparinase, heparin, and chlorate,



independently inhibit cell proliferation (55, 56). Cell surface syndecan is beginning to be recognized as a signaling receptor (57). Alterations in the phosphorylation state of syndecan may affect cell growth. It remains to be determined whether apoE alters syndecan phosphorylation and whether this is required for its antiproliferative effect. ApoE is also known to stimulate nitric oxide and cGMP production (9), which are antiproliferative (58). Although the role of this pathway in SMC proliferation was not determined (28), preliminary results showed that nitric oxide donor and cGMP can increase HSPG production in SMCs (not shown). Perlecan promoter has cAMP responsive elements (46). Thus, it is conceivable that increased cGMP or cAMP will induce transcription of perlecan mRNA through activation of specific transcription factors.

In summary, our data show that the antiproliferative effects of apoE are mediated by perlecan. We postulate that modulation of perlecan is a key step in regulating SMC growth. Factors that increase perlecan inhibit cell growth, whereas those that decrease perlecan stimulate cell growth.

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# **Exhibit C**

# The Inhibitory Effect of Apolipoprotein E4 on Neurite Outgrowth Is Associated with Microtubule Depolymerization\*

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Evidence is presented for the differential effects of two isoforms of apolipoprotein (apo) E, apoE3 and apoE4, on neurite outgrowth and on the cytoskeleton of neuronal cells (Neuro-2a) in culture. In the presence of a lipid source, apoE3 enhances and apoE4 inhibits neurite outgrowth. Immunocytochemical studies demonstrate that there is a higher concentration of apoE3 than apoE4 in both the cell bodies and neurites. Cells treated with apoE4 showed fewer microtubules and a greatly reduced ratio of polymerized to monomeric tubulin than did cells treated with apoE3. The effect of apoE4 on depolymerization of microtubules was shown by biochemical, immunocytochemical, and ultrastructural studies. The depolymerization of microtubules and the inhibition of neurite outgrowth associated with apoE4 suggest a mechanism whereby apoE4, which has been linked to the pathogenesis of Alzheimer's disease, may prevent normal neuronal remodeling from occurring later in life, when this neurodegenerative disorder develops.

Apolipoprotein (apo)<sup>1</sup> E is a 34-kDa protein component of lipoproteins that mediates their binding to the low density lipoprotein (LDL) receptor and to the LDL receptor-related protein (LRP) (1-4). Apolipoprotein E is a major apolipoprotein in the nervous system, where it is thought to redistribute lipoprotein cholesterol among the neurons and their supporting cells and to maintain cholesterol homeostasis (5-7). Apart from this function, apoE in the peripheral nervous system functions in the redistribution of lipids during regeneration (8-10).

There are three common isoforms of apoE (apoE2, apoE3, and apoE4) that are the products of three alleles ( $\epsilon$ 2,  $\epsilon$ 3, and  $\epsilon$ 4) at a single gene locus on chromosome 19 (11). Apolipoprotein E3, the most common isoform, has cysteine and arginine at positions 112 and 158, respectively, whereas apoE2 has cysteine at both of these positions and apoE4 has arginine at both (1, 12).

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<sup>1</sup> The abbreviations used are: apo, apolipoprotein; LDL, low density lipoprotein; LRP, LDL receptor-related protein; DRG, dorsal root ganglion; Neuro-2a, murine neuroblastoma; BALB/c, murine fibroblasts; DMEM, Dulbecco's modified Eagle's medium;  $\beta$ -VLDL,  $\beta$ -migrating very low density lipoproteins; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PIPES, 1,4-piperazinediethanesulfonic acid.

Accumulating evidence demonstrates that the apoE4 allele ( $\epsilon$ 4) is specifically associated with sporadic and familial late-onset Alzheimer's disease and is a major risk factor for the disease (13-16). In accord with these findings, apoE immunoreactivity is associated with both the amyloid plaques and the intracellular neurofibrillary tangles seen in postmortem examinations of brains from Alzheimer's disease patients (17, 18). The mechanism by which apoE4 might contribute to Alzheimer's disease is unknown. However, our recent data demonstrating that apoE4 stunts the outgrowth of neurites from dorsal root ganglion (DRG) neurons suggest that apoE may have a direct effect on neuronal development or remodeling (19, 20). In an extension of these previous studies, we have now examined the effects of the apoE isoforms on neurite outgrowth and on the cytoskeleton of Neuro-2a cells, a murine neuroblastoma cell line. Apolipoprotein E4 inhibits neurite outgrowth from these cells, and this isoform-specific effect is associated with depolymerization of microtubules.

## EXPERIMENTAL PROCEDURES

**Cell Lines**—Murine neuroblastoma (Neuro-2a) cells and murine fibroblasts (BALB/c) were obtained from American Type Culture Collection (Rockville, MD). Neuro-2a cells were maintained at 37 °C in a humidified 5% CO<sub>2</sub> incubator in Dulbecco's modified Eagle's medium-nutrient mixture (DMEM/F12; 50%:50%) containing 10% fetal bovine serum, penicillin, and streptomycin. For experiments, Neuro-2a cells were plated in this medium in 6-well plates at 25,000 cells/well. After 3-6 h of incubation, the medium was replaced with N2 medium (DMEM/F12 containing growth supplements) alone (21), N2 medium containing rabbit  $\beta$ -migrating very low density lipoproteins ( $\beta$ -VLDL) alone (40  $\mu$ g cholesterol/ml), or N2 medium containing  $\beta$ -VLDL and either purified human apoE3 (30  $\mu$ g/ml) or purified human apoE4 (30  $\mu$ g/ml), and the cells were incubated for an additional 48 h. BALB/c fibroblasts were maintained in DMEM containing 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in a humidified 7% CO<sub>2</sub> incubator. Experiments with BALB/c cells were performed in DMEM. Rabbit  $\beta$ -VLDL from cholesterol-fed animals were isolated as described (22), and human apoE was purified from the plasma of apoE3 and apoE4 homozygotes (23); biological activity was assessed by LDL receptor-binding assay (24).

**Quantitation of Neurite Outgrowth**—To assess neurite outgrowth, Neuro-2a cells were grown in test reagents and then nonspecifically stained with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (DiI) and fixed with 4% paraformaldehyde, as described previously (20). Neurons were imaged in fluorescence mode with a confocal laser scanning system (MRC-600, Bio-Rad), and the images were digitized with an Image-1/AT image analysis system (Universal Images, West Chester, PA) (20). The neuronal images were coded, and the neurite extension (the distance from the cell body to the end of the longest neurite) was measured for each neuron. Approximately 50-60 treatment-responsive cells (cells with at least one neurite longer than the diameter of the cell body) were measured for each treatment condition, and the data were calculated as the percent difference between each treatment group and the matched control (N2 medium alone) for each experiment. The percent differences for the different experiments then were averaged. The value for the N2 medium alone was set at 100%. Data are



presented as the mean  $\pm$  S.E. Statistics were done using Stat View II software.

**Immunocytochemistry**—Immunocytochemistry of apoE was performed on Neuro-2a cells and fibroblasts incubated for 48 h with  $\beta$ -VLDL and either apoE3 or apoE4. Cells were fixed for 3 days at 4 °C in PBS containing 3% paraformaldehyde and 0.1% glutaraldehyde and quenched with 150 mM sodium acetate in PBS containing 0.1% milk powder (quench buffer). The cells were further incubated for 15 min at room temperature in quench buffer with or without 0.5% Triton X-100. The cells were then washed with PBS containing 15 mM sodium acetate and 0.1% nonfat dry milk (wash solution) and incubated with a polyclonal antibody to apoE (GHE) at a concentration of 1:1000 in wash solution containing goat serum (1:50 dilution) for 1 h at room temperature followed by extensive washes with wash solution. The secondary antibody (anti-rabbit IgG conjugated to Texas Red, Vector Laboratories, Burlingame, CA) was incubated for 1 h before use at a 1:100 dilution in PBS containing 10% fetal bovine serum. The cells were washed with PBS and coverslipped, and serial optical sections ( $\sim 1 \mu\text{m}$  in thickness) were made using a confocal laser scanning microscope.

Immunocytochemistry to detect tubulin was performed on Neuro-2a cells and fibroblasts grown for 48 h in medium alone, with  $\beta$ -VLDL alone, or with  $\beta$ -VLDL and either apoE3 or apoE4. Following incubation with test reagents, the medium was aspirated, and the cells were washed twice with PBS. The cells were fixed for 1 h at room temperature in 10 mM HEPES, pH 7.2, containing 100 mM KCl, 3 mM  $\text{MgCl}_2$ , 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 0.5% Triton X-100, 2% paraformaldehyde, and 0.1% glutaraldehyde, followed by several quick washes with PBS. The cells were quenched with 0.05 M ammonium chloride in PBS for 5 min at room temperature and blocked for 1 h at room temperature with 3% bovine serum albumin (BSA) in PBS. Immunocytochemistry was performed for 45 min at room temperature, using a monoclonal antibody to  $\beta$ -tubulin (Boehringer Mannheim) at a concentration of 1  $\mu\text{g}/\text{ml}$  in PBS containing 1% BSA. Following incubation, the cells were washed five times with PBS containing 0.1% BSA and then incubated for 30 min in the dark with goat anti-mouse IgG (Zymed Laboratories Inc., South San Francisco, CA) conjugated to fluorescein isothiocyanate (10  $\mu\text{l}/\text{ml}$ ) in PBS containing 1% BSA. Cells were coverslipped, and optical sections of 0.5  $\mu\text{m}$  thickness were made using a Bio-Rad MRC-600 confocal laser scanning microscope; the sections were overlaid to obtain a composite image.

For localization of actin, the medium was aspirated, and the cells were washed twice with PBS. Cells were fixed with 3% paraformaldehyde in PBS, washed twice with PBS, and permeabilized for 5 min at room temperature with 0.25% Triton X-100 in PBS containing 1% BSA. The permeabilized cells were washed twice with PBS and incubated for 30 min at room temperature in PBS containing 5 units/ml of rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR). The cells were washed twice with PBS and coverslipped, and optical sections were made as described above.

In immunocytochemistry experiments, 15–20% of the cells did not respond to the treatments and were similar to control neurons. These cells may represent cells injured during plating.

**$^{125}\text{I}$ -ApoE Binding Assay**—The cells were grown in 12-well plates until they reached half of maximal confluence. Cells were washed with medium and incubated with  $\beta$ -VLDL (40  $\mu\text{g}$  cholesterol/ml) along with 30  $\mu\text{g}/\text{ml}$  of either  $^{125}\text{I}$ -apoE3 or  $^{125}\text{I}$ -apoE4 for 48 h. Following incubation, the medium was removed and the cells were washed four times with PBS containing 0.2% BSA at 4 °C. The cells were solubilized with 0.1 N NaOH and assayed for protein, and radioactivity was determined by gamma counting. Apolipoprotein E was iodinated using Bolton-Hunter reagent (Amersham Corp.) according to the manufacturer's instructions. The average specific activity was 50 counts/min/ng apoE.

**Electron Microscopy of Neuro-2a Cells**—Neuro-2a cells were incubated with test reagents as described above. Following incubation, the cells were lifted from the plates using 0.05% trypsin and 0.5 mM EDTA and pelleted by centrifugation. Cells were fixed for 1 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed for 1 h in 2%  $\text{OsO}_4$ . The cells were dehydrated, embedded in Epon 812, sectioned (80 nm) using a Reichert Ultracut E, and stained with uranyl acetate and lead citrate. The cells were photographed using a JEOL CX-100II electron microscope. Consistent results were obtained in three independent experiments performed with fresh preparations of apoE and  $\beta$ -VLDL.

**Binding of  $^{125}\text{I}$ -ApoE to Microtubules**—Neuro-2a cells in a 100-mm plate were grown to confluence as described above, scraped from the plate in PBS, and lysed by sonication in PME buffer, pH 7.2 (80 mM PIPES, 1 mM  $\text{MgCl}_2$ , 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5  $\mu\text{g}/\text{ml}$  leupeptin), containing 1 mM GTP. The solution was centrifuged

and pelleted cell debris discarded. The supernatant was incubated at 37 °C for 1 h to facilitate tubulin polymerization. The resultant pellet of microtubules was resuspended in PME buffer, aliquoted into microfuge tubes, and incubated at 37 °C with either  $^{125}\text{I}$ -apoE3 (2  $\mu\text{g}/\text{ml}$ ) or  $^{125}\text{I}$ -apoE4 (2  $\mu\text{g}/\text{ml}$ ). Following incubation, the samples were centrifuged for 1 h at  $100,000 \times g$  to separate the free  $^{125}\text{I}$ -apoE from that bound to microtubules. The radioactivity associated with the supernatant and pellet was estimated by gamma counting.

**Immunoblotting of Tubulin**—Neuro-2a cells were incubated with test reagents, and cell extracts were prepared as described above. An aliquot of each extract containing an equal amount of protein was centrifuged through a sucrose cushion (PME + 20% sucrose) at  $100,000 \times g$  for 1 h at 37 °C to separate the microtubules (polymeric tubulin preparation) from the tubulin (monomeric tubulin preparation). The monomeric tubulin preparation was polymerized for 1 h at 37 °C by incubating the sample in PME buffer containing 20% sucrose and 1 mM GTP, followed by centrifugation as described above (25). The polymeric tubulin preparation was subjected to a temperature-dependent depolymerization-polymerization cycle to remove cellular debris, followed by centrifugation to obtain the microtubules. Aliquots of the cell extract (total tubulin) and the monomeric and polymeric tubulin preparations were subjected to 12% SDS-polyacrylamide gel electrophoresis under reducing conditions and immunoblotted using a monoclonal antibody to  $\alpha$ -tubulin (ICN, Irvine, CA) at a dilution of 1:100, as described by the manufacturer. The tubulin bands in the immunoblots were quantitated by densitometry (Ambis Systems, San Diego, CA). The value obtained with N2 medium alone was set at 100%, and the data were calculated as the percent difference between each treatment group and the matched control (N2 medium alone) for each experiment. The percent differences for the different experiments then were averaged. Data are presented as the mean  $\pm$  S.E. Statistics were done using Stat View II software.

**Other Assays and Methods**—Protein assay was performed as described (26). The amount of cholesterol in cells incubated for 48 h with  $\beta$ -VLDL (40  $\mu\text{g}$  cholesterol/ml) and human apoE3 or apoE4 (30  $\mu\text{g}/\text{ml}$ ) was assayed using a commercially available kit (Monotest, Boehringer Mannheim). The [ $^3\text{H}$ ]thymidine incorporation assay was performed with Neuro-2a cells incubated with  $\beta$ -VLDL and apoE3 or apoE4 using a previously published procedure (27). The lactate dehydrogenase assay was performed as described (28). The DiI-labeled  $\beta$ -VLDL uptake was performed as described (19).

## RESULTS

**Differential Effects of ApoE3 and ApoE4 on Neurite Outgrowth**—Our previous studies examined the effects of apoE and lipoproteins on the outgrowth of neurites from primary rabbit DRG neurons *in vitro* (19, 20). The addition of purified human apoE3, together with rabbit  $\beta$ -VLDL (cholesterol-rich lipoproteins), increased neurite extension from these peripheral nervous system neurons, whereas apoE4 when added to the cells together with  $\beta$ -VLDL inhibited neurite outgrowth.

In this study, we found a similar differential effect of apoE3 and apoE4 on the outgrowth of neurites from Neuro-2a cells. In the presence of  $\beta$ -VLDL, apoE3 and apoE4 had dramatic isoform-specific effects on neurite outgrowth from Neuro-2a cells, as assessed by phase contrast microscopy (Fig. 1). Incubation of the cells with  $\beta$ -VLDL (Fig. 1B) stimulated neurite outgrowth slightly as compared with cells grown in N2 medium alone (Fig. 1A); however, a more dramatic effect was seen with the addition of apoE. Cells incubated with apoE3 and  $\beta$ -VLDL (Fig. 1C) had more neurite extension than cells incubated with  $\beta$ -VLDL, whereas cells incubated with apoE4 and  $\beta$ -VLDL had less neurite extension (Fig. 1D). These observations were confirmed when neurite outgrowth was quantitated using an image analysis system (Fig. 2). Incubation of the cells with  $\beta$ -VLDL enhanced neurite extension, as compared with cells maintained in N2 medium alone (Fig. 2). Addition of human apoE3 with  $\beta$ -VLDL further increased the extension ( $p < 0.005$ ), whereas human apoE4 along with  $\beta$ -VLDL significantly reduced neurite extension as compared with the extension observed from cells incubated with  $\beta$ -VLDL ( $p < 0.001$ ).

Three different studies were performed to rule out a general toxic effect of apoE4 on neurons. We assayed lactate dehydro-



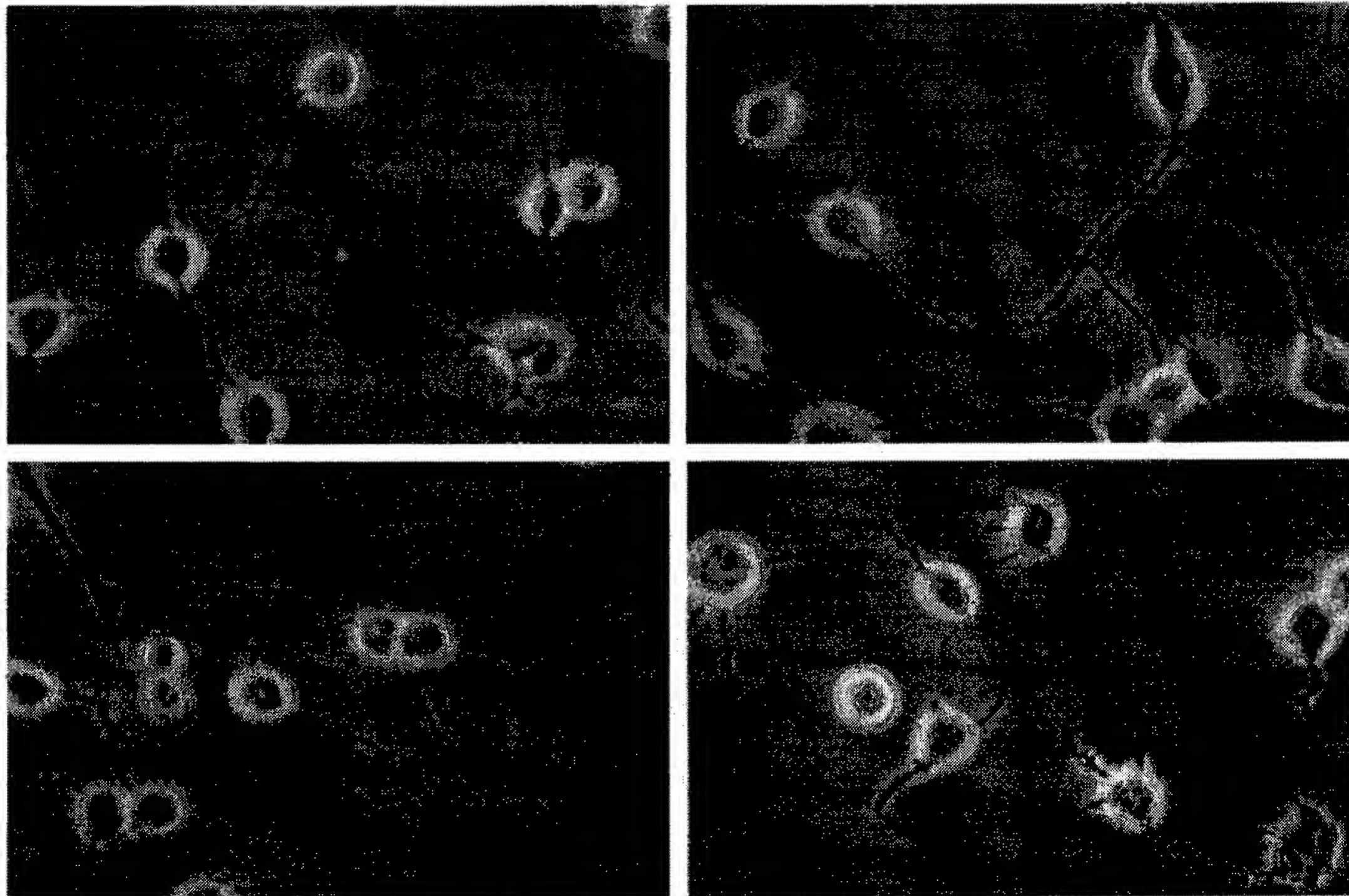


FIG. 1. **Effect of apoE on neurite outgrowth from Neuro-2a cells.** Neuro-2a cells were grown for 2 days in N2 medium alone (A), in N2 medium with  $\beta$ -VLDL (40  $\mu$ g of cholesterol/ml) (B), or in N2 medium with  $\beta$ -VLDL (40  $\mu$ g of cholesterol/ml) and 30  $\mu$ g/ml of either human apoE3 (C) or human apoE4 (D). Cells were photographed using a phase contrast microscope. Scale bar = 25  $\mu$ m.

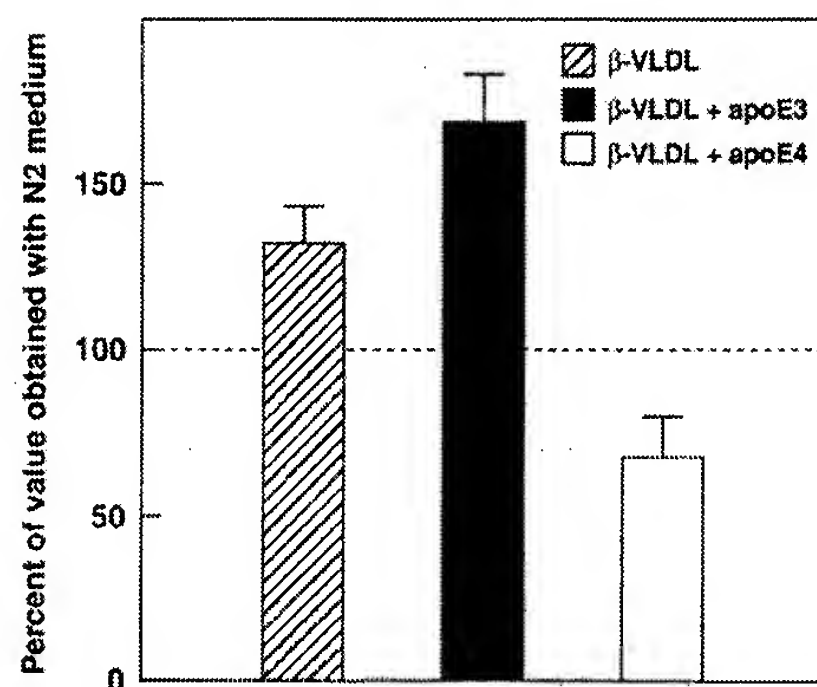


FIG. 2. **Quantitation of the effect of apoE on neurite extension from Neuro-2a cells.** Neuro-2a cells were incubated with test reagents as described in the legend to Fig. 1. Neurite extension then was measured for 50–60 neurons from each group as described under “Experimental Procedures.” Data were calculated as the percent difference between each treatment group and the matched control (N2 medium alone) for each experiment. The percent differences for the various experiments then were averaged. The value for the N2 medium alone was set at 100% (dashed line). Data are presented as the mean  $\pm$  S.E.

genase activity, a commonly used indicator of cell death, measured thymidine incorporation into DNA as an indication of cell replication, and examined the ability of the cells to develop neurites following incubation with apoE4 and  $\beta$ -VLDL. No significant differences in lactate dehydrogenase activity (apoE3 +  $\beta$ -VLDL,  $212 \pm 26.2$  units/ml; apoE4 +  $\beta$ -VLDL,  $205 \pm 22$  units/ml) or [ $^3$ H]thymidine incorporation into DNA (apoE3 +  $\beta$ -VLDL,  $17.37 \pm 1.66$  counts/min  $\times 10^6$ ; apoE4 +  $\beta$ -VLDL,  $18.13 \pm 1.42$  counts/min  $\times 10^6$ ) were seen with cells incubated with  $\beta$ -VLDL and either apoE3 or apoE4. The inhibitory effect of apoE4 was reversible, since removal of apoE4 and  $\beta$ -VLDL

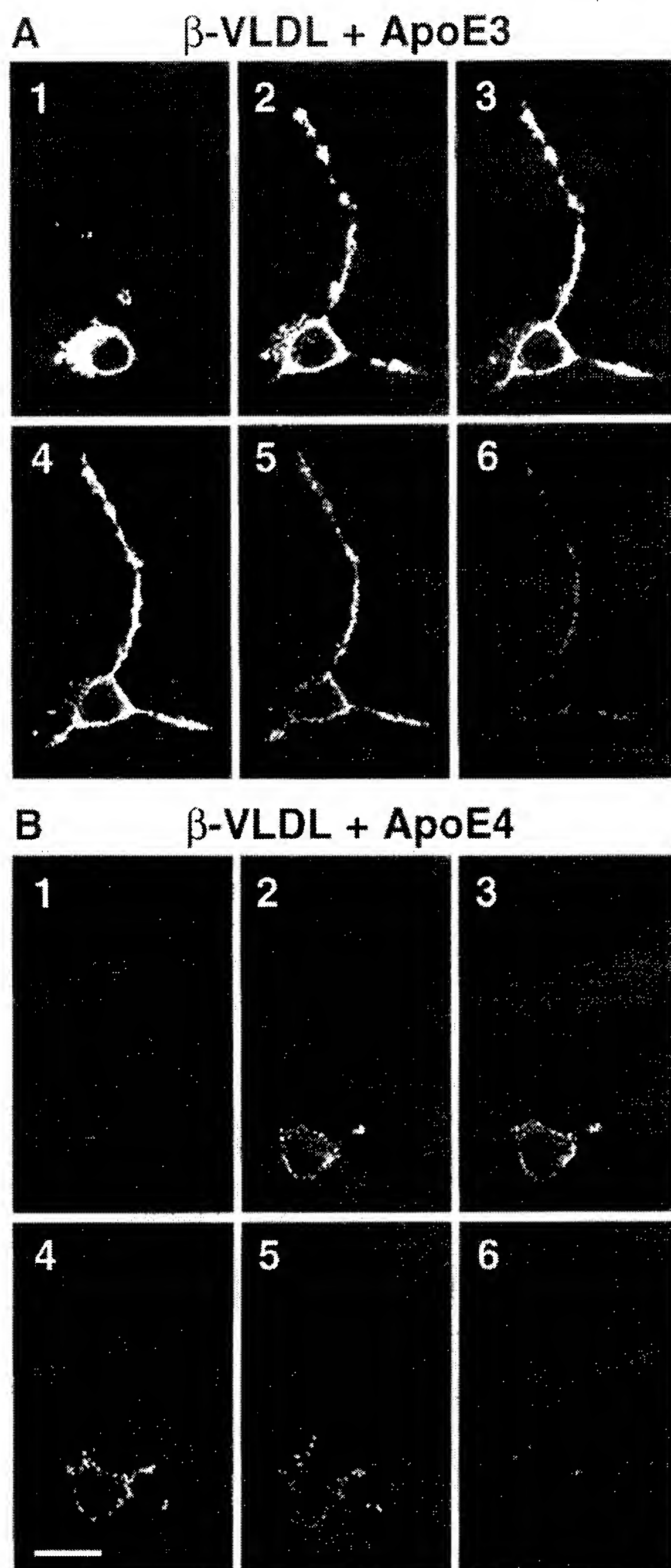
from the cells in culture and addition of medium alone or medium containing  $\beta$ -VLDL resulted in normal outgrowth (data not shown). These findings taken together demonstrate that the inhibitory effect of apoE4 on neurite outgrowth is not due to a cytotoxic effect of apoE4 on the cells.

To determine if lipoproteins were required for the differential effects of apoE3 and apoE4, the cells were incubated with free apoE for 48 h and the effects on neurite outgrowth examined. In the absence of lipoprotein, neither apoE3 nor apoE4 had an effect on neurite extension (data not shown). This result suggests that receptor-mediated endocytosis of apoE is necessary for the differential effects on neurite outgrowth, as previous studies have shown that apoE is a ligand for the LDL receptor and the LRP only when it is present on lipoproteins (1–3, 29).

**Metabolism and Localization of ApoE3 and ApoE4 in Neuro-2a Cells**—In several nonneuronal cell lines it has been demonstrated that apoE-containing lipoproteins follow a classic receptor-mediated endocytotic pathway by which the ligands are delivered to lysosomes where they are degraded (1–3, 12). Both apoE3- and apoE4-containing lipoproteins exhibit similar binding activity in nonneuronal cells (1, 22, 30). However, recent studies showing apoE immunoreactivity in the cytoplasm of neurons (31) suggest differences between neuronal and nonneuronal cells in the metabolism of apoE.

We first examined the ability of  $\beta$ -VLDL together with either apoE3 or apoE4 to deliver lipids to the cells. Incubation of the cells with  $\beta$ -VLDL and apoE3 or apoE4 for 48 h did in fact lead to similar levels of lipid accumulation (apoE3 +  $\beta$ -VLDL,  $115 \pm 3.9$   $\mu$ g cholesterol/mg of cell protein; apoE4 +  $\beta$ -VLDL,  $121 \pm 5.8$   $\mu$ g cholesterol/mg of cell protein), suggesting that the stimulation of  $\beta$ -VLDL uptake was similar with the two apoE isoforms. In comparison, Neuro-2a cells accumulated  $20 \pm 0.9$  and  $86 \pm 2.6$   $\mu$ g of cholesterol/mg of cell protein when incubated

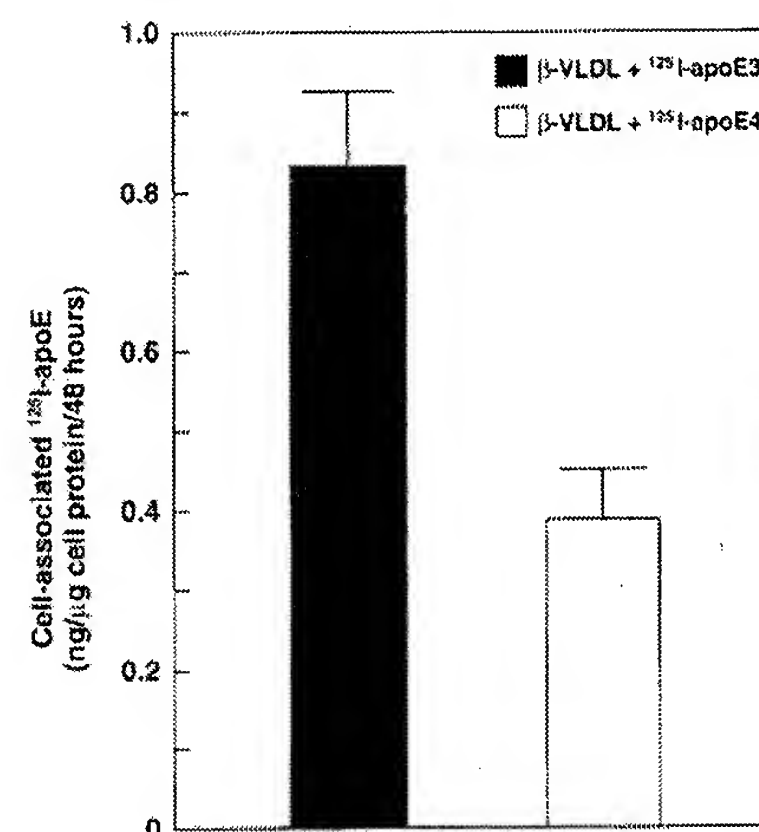




**FIG. 3. Immunocytochemical localization of apoE in Neuro-2a cells.** Neuro-2a cells were grown for 2 days in medium containing  $\beta$ -VLDL (40  $\mu$ g of cholesterol/ml) together with 30  $\mu$ g/ml of either human apoE3 (A) or human apoE4 (B). Immunocytochemistry was performed as described under "Experimental Procedures." Serial optical sections ( $\sim 1$   $\mu$ m in thickness) were made from the top (section 1) to the bottom (section 6) of the cells using a confocal laser scanning microscope. Scale bar = 15  $\mu$ m.

with N2 medium and  $\beta$ -VLDL alone, respectively. Examination of the uptake of fluorescently labeled  $\beta$ -VLDL in the presence of either apoE3 or apoE4 supported the conclusion that apoE3 and apoE4 stimulated  $\beta$ -VLDL uptake similarly.

To determine whether apoE3 and apoE4 were processed



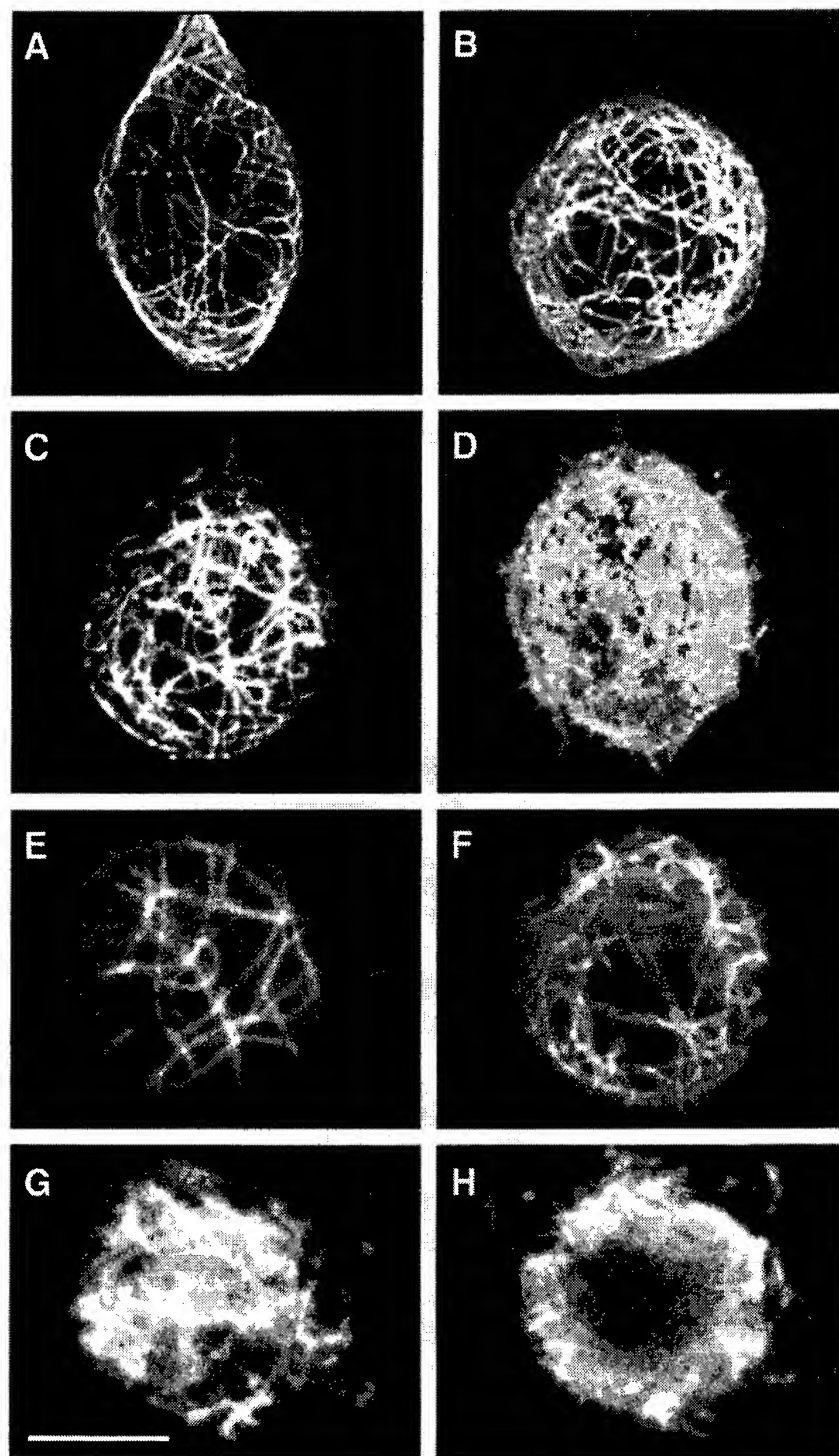
**FIG. 4. Cell association of  $^{125}$ I-apoE with Neuro-2a cells.** Neuro-2a cells were grown for 2 days in medium containing  $\beta$ -VLDL (40  $\mu$ g of cholesterol/ml) together with 30  $\mu$ g/ml of either  $^{125}$ I-apoE3 or  $^{125}$ I-apoE4. Following incubation, the radioactivity associated with the cells (representing bound and internalized apoE) was determined as described under "Experimental Procedures." The experiment was repeated five times, each time with a fresh preparation of  $^{125}$ I-apoE and  $\beta$ -VLDL. The data are presented as the mean  $\pm$  S.E.

differently by neurons and by fibroblasts, we incubated Neuro-2a cells or murine fibroblasts with apoE3 or apoE4 in the presence of  $\beta$ -VLDL and examined the accumulation of apoE in the cells. Immunocytochemical detection of apoE in Neuro-2a cells incubated with  $\beta$ -VLDL and either apoE3 or apoE4 revealed that both isoforms were present within neurons (Fig. 3). There was, however, a substantial difference in the intensity of reactivity of apoE3 (Fig. 3A) and apoE4 (Fig. 3B). Apolipoprotein E3 was present both in the cell body and in the neurites at a substantially higher concentration than was apoE4 (Fig. 3, A and B). Both apoE3 and apoE4 were observed in nearly all serial optical sections made throughout the cell, suggesting that apoE was intracellular (Fig. 3, A and B).

The possibility that apoE was localized intracellularly was examined using two additional approaches. First, Neuro-2a cells were incubated with  $\beta$ -VLDL and either apoE3 or apoE4 and treated with suramin (a polyanion known to remove lipoproteins nonspecifically bound to the cell surface and specifically bound to their receptors), followed by immunocytochemistry for apoE. Treatment with suramin did not significantly reduce the apoE immunoreactivity associated with the cells. Second, immunocytochemical studies were performed in cells that were not permeabilized to permit access of antibody to the cytoplasm. No immunoreactivity of apoE was observed in non-permeabilized cells incubated with  $\beta$ -VLDL together with either apoE3 or apoE4. These results demonstrated that the apoE was intracellular. The observed intracellular accumulation of apoE was unexpected, since lipoproteins and their apoproteins, when internalized by nonneuronal cell types, are rapidly degraded. To determine if the accumulation of apoE was specific to neurons, we performed similar immunocytochemistry experiments in murine fibroblasts. In these studies no apoE immunoreactivity was observed in the fibroblasts incubated with  $\beta$ -VLDL and either apoE3 or apoE4, suggesting either that apoE enters neurons and fibroblasts through different pathways or that in neurons apoE, especially apoE3, can escape lysosomal degradation. These results demonstrate that apoE3 is retained in Neuro-2a cells to a greater extent than apoE4 and that the metabolism of apoE in neuronal and non-neuronal cells is different.

The differences observed in the accumulation of apoE3 and apoE4 were confirmed by incubating the neurons with  $^{125}$ I-





**FIG. 5. Immunocytochemical localization of tubulin in Neuro-2a cells.** Neuro-2a cells were incubated with test reagents as described in the legend to Fig. 1. Immunocytochemistry was performed using a monoclonal antibody to  $\beta$ -tubulin and visualized using a fluorescein-labeled secondary antibody. Optical sections (0.5  $\mu$ m in thickness) were made using a confocal microscope, and sections were overlaid to obtain a reconstructed image. Reconstructed images of cells incubated in medium alone (A), in medium containing  $\beta$ -VLDL (40  $\mu$ g cholesterol/ml) (B), or in medium containing  $\beta$ -VLDL together with 30  $\mu$ g/ml of either human apoE3 (C) or human apoE4 are shown (D). Representative individual optical sections near the top (E and G) and center (F and H) of cells incubated with  $\beta$ -VLDL and either apoE3 (E and F) or apoE4 (G and H) also are shown. Scale bar = 7  $\mu$ m.

labeled apoE and  $\beta$ -VLDL at 37  $^{\circ}$ C for 48 h, and the amount of cell-associated apoE (bound and internalized) was quantitated. A differential accumulation of  $^{125}$ I-apoE was observed, with twice as much  $^{125}$ I-apoE3 as  $^{125}$ I-apoE4 being associated with the cells at the end of the incubation period (Fig. 4).

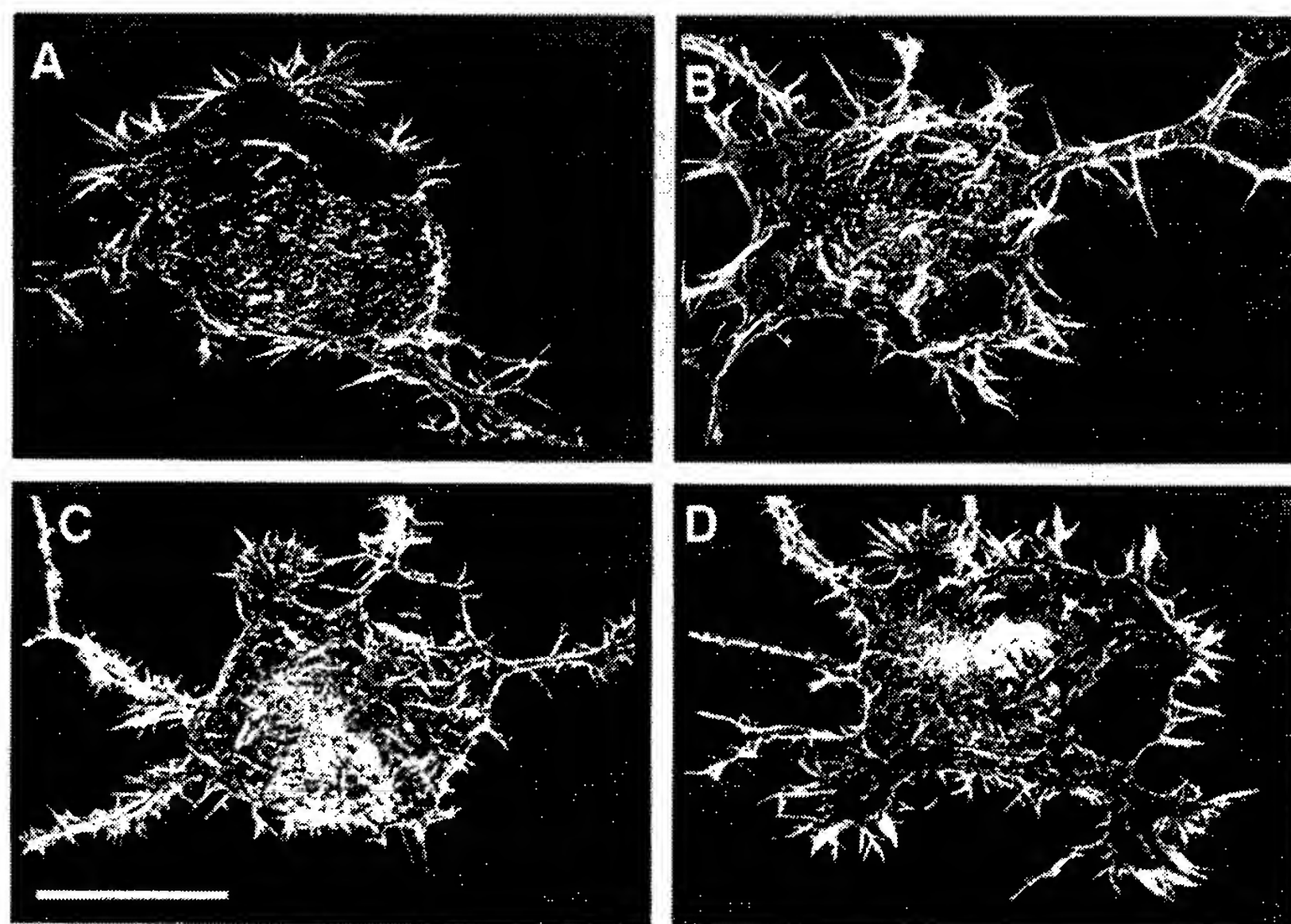
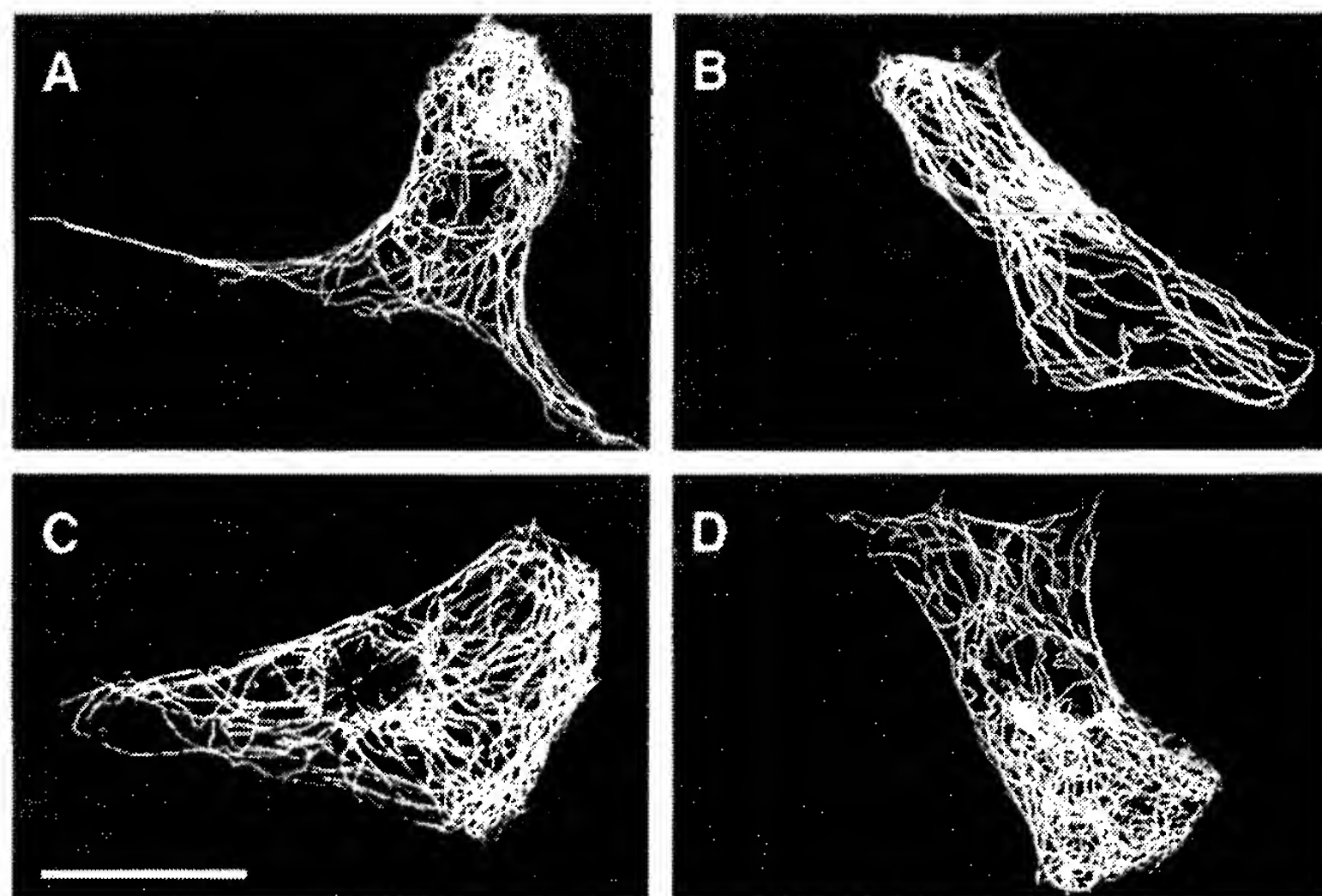
**Effects of ApoE3 and ApoE4 on the Microtubular Architecture of Neuro-2a Cells**—Microtubules and their associated proteins play a crucial role in neurite outgrowth (32–37). Because apoE accumulates in the neurons and affects neurite outgrowth, we hypothesized that it also might affect tubulin polymerization and microtubule formation. Immunocytochemistry and electron microscopy were used to examine the cells incubated in medium alone, in medium containing  $\beta$ -VLDL, and in medium containing  $\beta$ -VLDL together with either apoE3 or apoE4. In cells grown in medium alone (Fig. 5A), with  $\beta$ -VLDL alone (Fig.

5B), or with  $\beta$ -VLDL and apoE3 (Fig. 5C), a well-formed network of microtubules was observed by immunocytochemistry. In contrast, in cells incubated with apoE4 and  $\beta$ -VLDL, few well-organized microtubules were present (Fig. 5D). The tubulin immunoreactivity in the apoE4-treated cells was diffuse throughout the cell body and in numerous cell-surface projections (consistent with the presence of monomeric tubulin) (38).

The photomicrographs in Fig. 5 (A–D) are composites of all optical sections. However, we also analyzed individual optical sections visualizing various levels through the cells (Fig. 5, E–H) to ensure that the presence of microtubules in the apoE4-treated cells was not obscured by the diffuse tubulin staining. In cells treated with  $\beta$ -VLDL and apoE4 (Fig. 5, G and H), none of the optical sections contained well-formed microtubules, whereas in cells incubated with  $\beta$ -VLDL and apoE3 (Fig. 5, E



**FIG. 6. Immunocytochemical localization of tubulin in fibroblasts.** BALB/c fibroblasts were incubated for 2 days in medium alone (A), in medium containing  $\beta$ -VLDL (40  $\mu$ g cholesterol/ml) (B), or in medium containing  $\beta$ -VLDL together with 30  $\mu$ g/ml of either human apoE3 (C) or human apoE4 (D). Immunocytochemistry for  $\beta$ -tubulin was performed as described in the legend to Fig. 5. Scale bar = 7  $\mu$ m.



**FIG. 7. Localization of actin in Neuro-2a cells.** Neuro-2a cells were incubated with test reagents as described in the legend to Fig. 1. Actin was detected using rhodamine-phalloidin. Optical sections (1  $\mu$ m in thickness) were made using a confocal microscope, and sections were overlaid to obtain a reconstructed image. Reconstructed images of cells incubated in medium alone (A), in medium containing  $\beta$ -VLDL (40  $\mu$ g cholesterol/ml) (B), or in medium containing  $\beta$ -VLDL together with 30  $\mu$ g/ml of either human apoE3 (C) or human apoE4 are shown (D). Scale bar = 8  $\mu$ m.

and F'), numerous microtubules were seen in nearly all of the sections. When similar experiments were performed with murine fibroblasts, no significant difference in microtubular morphology was observed among cells treated with medium alone (Fig. 6A), medium containing  $\beta$ -VLDL (Fig. 6B), or medium containing  $\beta$ -VLDL together with either apoE3 (Fig. 6C) or apoE4 (Fig. 6D).

Additional experiments were performed to determine if apoE4 disrupts other cytoskeletal structures, such as actin filaments. As shown in Fig. 7, no significant difference in actin morphology was evident among Neuro-2a cells from the four treatment conditions when actin was detected using rhodamine-phalloidin.

To confirm the fluorescence microscopy data and to examine the effect of apoE on the microtubular architecture in the neurites of Neuro-2a cells, we performed electron microscopic studies. Long parallel arrays, identified by morphologic appearance and size (~22 nm in width) as microtubules, were present in cells grown in medium alone (Fig. 8A),  $\beta$ -VLDL alone (Fig. 8B), and  $\beta$ -VLDL and apoE3 (Fig. 8C). In contrast,

in cells treated with apoE4 and  $\beta$ -VLDL, only a few fragments of microtubules were observed both in the cell body and neurites (Fig. 8D). All of these data taken together demonstrate that the differences in neurite outgrowth induced by apoE3 and apoE4 are associated with differences in microtubular formation and suggest that apoE4 depolymerizes microtubules in neuronal cells.

**Effects of ApoE on the Polymerization State of Tubulin in Neuro-2a Cells**—Further studies were performed to determine whether apoE associates with microtubules and to determine by biochemical assays if the amount of polymerized tubulin differed in the cells treated with apoE3 or apoE4. To determine if apoE associates with microtubules, total tubulin was extracted from the cells grown in medium alone and polymerized *in vitro*. This partially purified preparation, which contained microtubules and microtubule-associated proteins, was tested for its ability to bind apoE3 and apoE4. When  $^{125}$ I-apoE was incubated with the partially purified microtubules from Neuro-2a cells, both  $^{125}$ I-apoE3 and  $^{125}$ I-apoE4 bound to the microtubules and/or their associated proteins (Fig. 9). However, an isoform-specific difference was



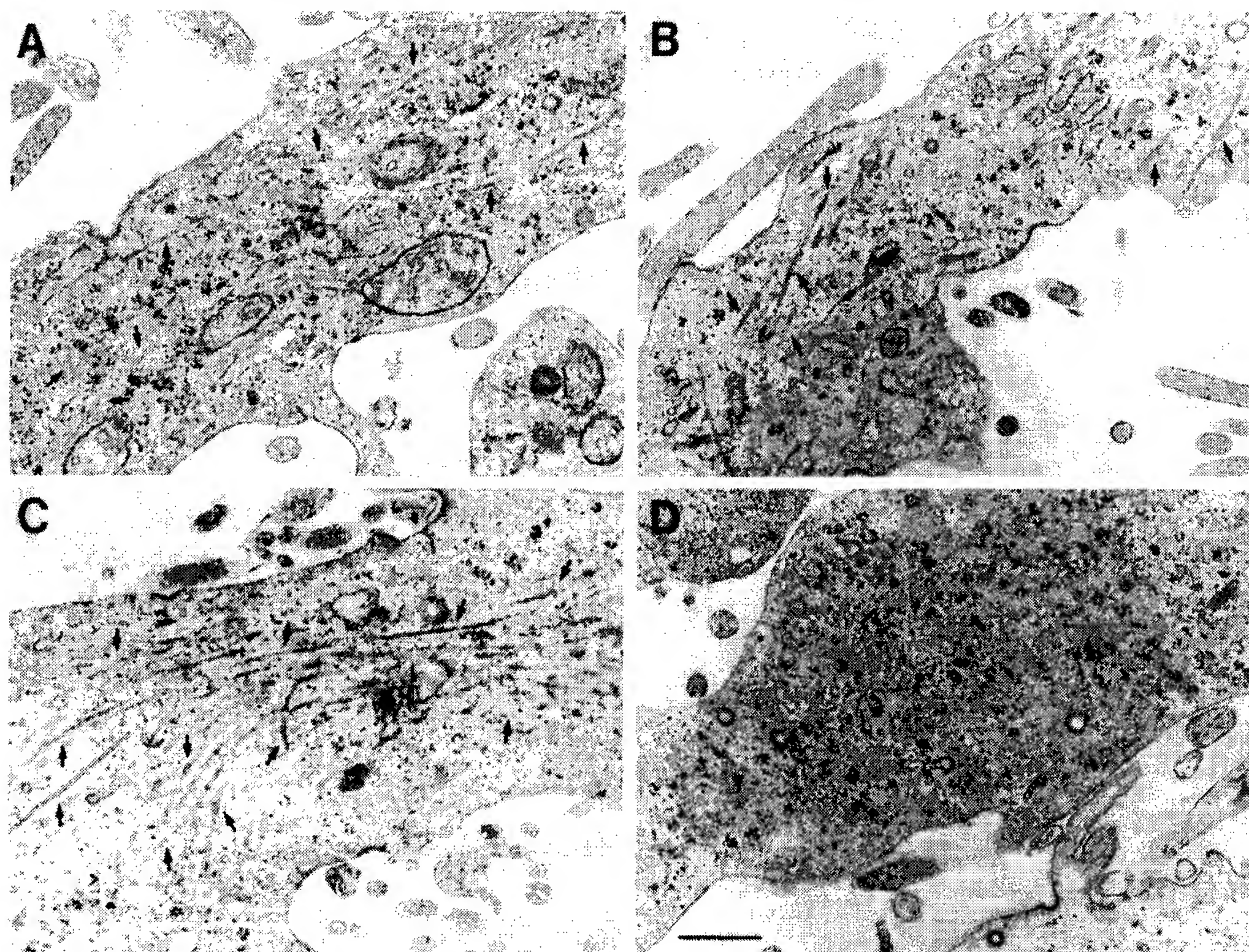


FIG. 8. **Electron microscopy of microtubules in Neuro-2a cells.** Neuro-2a cells were incubated for 2 days in medium alone (A), in medium containing  $\beta$ -VLDL (40  $\mu$ g cholesterol/ml) (B), or in medium containing  $\beta$ -VLDL together with 30  $\mu$ g/ml of either human apoE3 (C) or human apoE4 (D). Following incubation, electron microscopy was performed, as described under "Experimental Procedures," to detect microtubules (arrows) in the neurites. Scale bar = 0.5  $\mu$ m.

observed, with more  $^{125}$ I-apoE3 than  $^{125}$ I-apoE4 bound to the crude microtubule preparation.

The effect of apoE on the ratio of polymerized to monomeric tubulin was examined biochemically. Total tubulin, monomeric tubulin, and polymerized tubulin were extracted from Neuro-2a cells incubated under the various conditions and then were quantitated by Western blotting and densitometry. The amounts of total tubulin in extracts from the cells grown under the four treatment conditions were similar (Fig. 10A). Incubation of cells with N2 medium containing  $\beta$ -VLDL did not significantly affect the quantity of monomeric or polymeric forms of tubulin as compared with cells grown in N2 medium alone (Fig. 10A). However, a dramatic isoform-specific difference in the polymerization state of the microtubules was observed when the cells were grown in medium containing  $\beta$ -VLDL and apoE. In contrast to cells treated with apoE3 and  $\beta$ -VLDL, incubation of the cells with apoE4 and  $\beta$ -VLDL resulted in an increase in the amount of monomeric tubulin and a decrease in the amount of polymeric tubulin (microtubules).

Quantitation of the immunoblots from three independent experiments by densitometry revealed that the incubation of Neuro-2a cells with  $\beta$ -VLDL resulted in a slight increase in the amount of monomeric and polymeric tubulin (Fig. 10B) as compared with cells grown in N2 medium alone. However, cells incubated with  $\beta$ -VLDL plus apoE3 had a significant decrease in monomeric tubulin and a significant increase in polymeric tubulin as compared with cells grown in  $\beta$ -VLDL alone ( $p < 0.001$ ) (Fig. 10B). On the other hand, the opposite effect was observed in cells treated with apoE4 and  $\beta$ -VLDL, as monomeric tubulin increased significantly and polymeric tubulin decreased ( $p < 0.001$ ). These biochemical results confirm and

extend the ultrastructural studies and suggest that apoE alters the state of tubulin polymerization in an isoform-specific manner.

#### DISCUSSION

The present study demonstrates that the isoform-specific effect of apoE in association with a lipid source on neurite outgrowth that was previously seen in rabbit DRG neurons also is observed in Neuro-2a cells, a murine neuroblastoma cell line derived from the central nervous system (19, 20). Furthermore, this study demonstrates that the isoform-specific effect of apoE on neurite outgrowth correlates with a differential accumulation of apoE3 versus apoE4 within the neurons and with a differential effect of the apoE3 and apoE4 on cellular microtubules.

Previous studies of nonneuronal cells have shown that apoE-containing lipoproteins are taken up and degraded by receptor-mediated endocytosis. Apolipoprotein E3- and apoE4-containing lipoproteins have a similar binding affinity and cause a similar degree of lipoprotein internalization (1, 22, 30). The LDL receptor and the LRP have been implicated in this process (1–3, 29). Neuronal cells possess both of these major receptors (19, 39, 40), and apoE3-induced neurite extension in DRG neurons has been suggested to be mediated, at least in part, by the LRP (19).

In the present study, using immunocytochemistry of apoE, we have observed a differential accumulation of apoE3 and apoE4 in Neuro-2a cells. Apolipoprotein E3, incubated with the cells together with  $\beta$ -VLDL, accumulated widely throughout the cell bodies and the neurites in a diffuse pattern, suggesting that the internalized apoE3 is not restricted to a specific organelle. Confocal microscopy revealed that the apoE3 is intra-



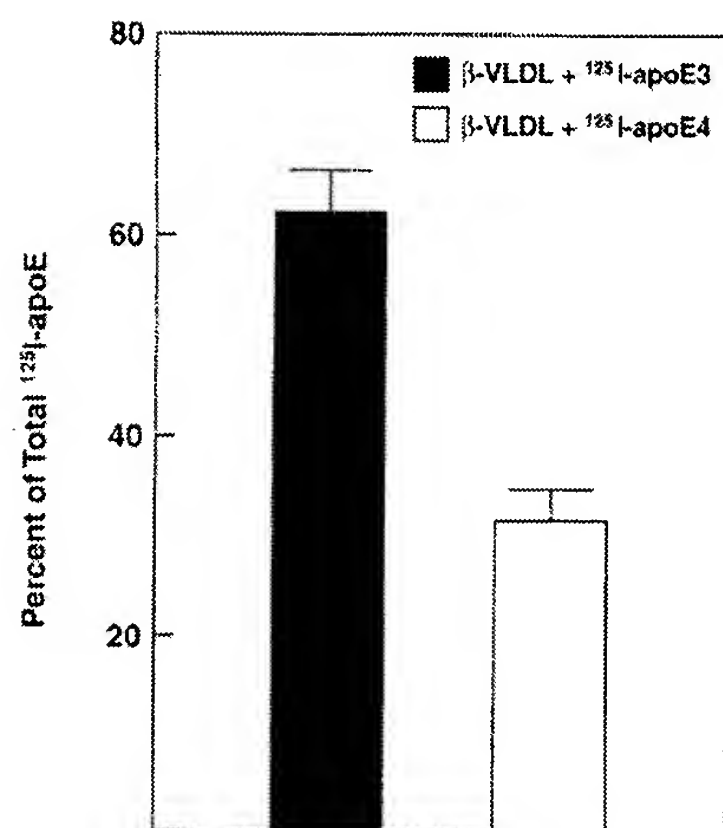


FIG. 9. Binding of <sup>125</sup>I-apoE to partially purified microtubules *in vitro*. Partially purified microtubules were prepared from Neuro-2a cells grown in N2 medium, as described under "Experimental Procedures." Aliquots of microtubular preparations were incubated with <sup>125</sup>I-apoE3 or <sup>125</sup>I-apoE4 (2 μg/ml), followed by centrifugation to separate the free <sup>125</sup>I-apoE from that bound to microtubules. The radioactivity associated with the supernatant and pellet was determined, and the percentage of total radioactivity in the pellet was calculated. The data are the mean ± S.E. for three independent experiments.

cellular, an observation confirmed by the fact that the pattern of staining did not change when the cells were treated with suramin to remove surface-bound apoE and by the fact that the immunoreactivity was observed only after membrane permeabilization to allow the antibodies to enter the cells. In comparison to apoE3, less apoE4 accumulates within the Neuro-2a cells, even though equal amounts of lipoprotein-derived lipid are delivered to the cells. The data suggest a differential processing of apoE3 and apoE4, resulting in a greater accumulation of apoE3. How apoE escapes lysosomal degradation remains to be determined. The ability of apoE to escape lysosomal degradation is supported by the recent observations of Han *et al.* (31), which suggest that apoE is present in the cytoplasm of human neurons.

The differential effect of the apoE isoforms on neurite extension and their differential intracellular accumulation suggested that apoE might alter the neuronal cytoskeleton, specifically the microtubular system. Microtubules have been shown to have several important functions in neurons, including the development and maintenance of neuronal polarity, neurite extension, and retraction, the transport of macromolecules, and the release of neurotransmitters (32–37, 41, 42). In fact, microtubular stability is linked to several neurodegenerative disorders, including Alzheimer's disease (43). We have shown by three different criteria that apoE3 and apoE4, when incubated with the cells together with β-VLDL, have differential effects on microtubular structure. The apoE3 clearly supports microtubule formation in the Neuro-2a cells, whereas apoE4 is associated with microtubular depolymerization. By immunocytochemistry, the cells incubated with apoE3 along with β-VLDL displayed an extensive microtubular system as visualized using an anti-tubulin antibody. In the apoE4-treated cells, the microtubules were poorly formed and revealed a diffuse immunoreactivity to tubulin, suggesting the depolymerization of the microtubules. These observations were confirmed by electron microscopy of the Neuro-2a cells. Furthermore, quantitation of monomeric and polymeric tubulin extracted from the Neuro-2a cells revealed that incubation of the cells with apoE3 along with β-VLDL resulted in a reduction in monomeric tubulin and an increase in polymeric tubulin, whereas the opposite results were observed with apoE4.

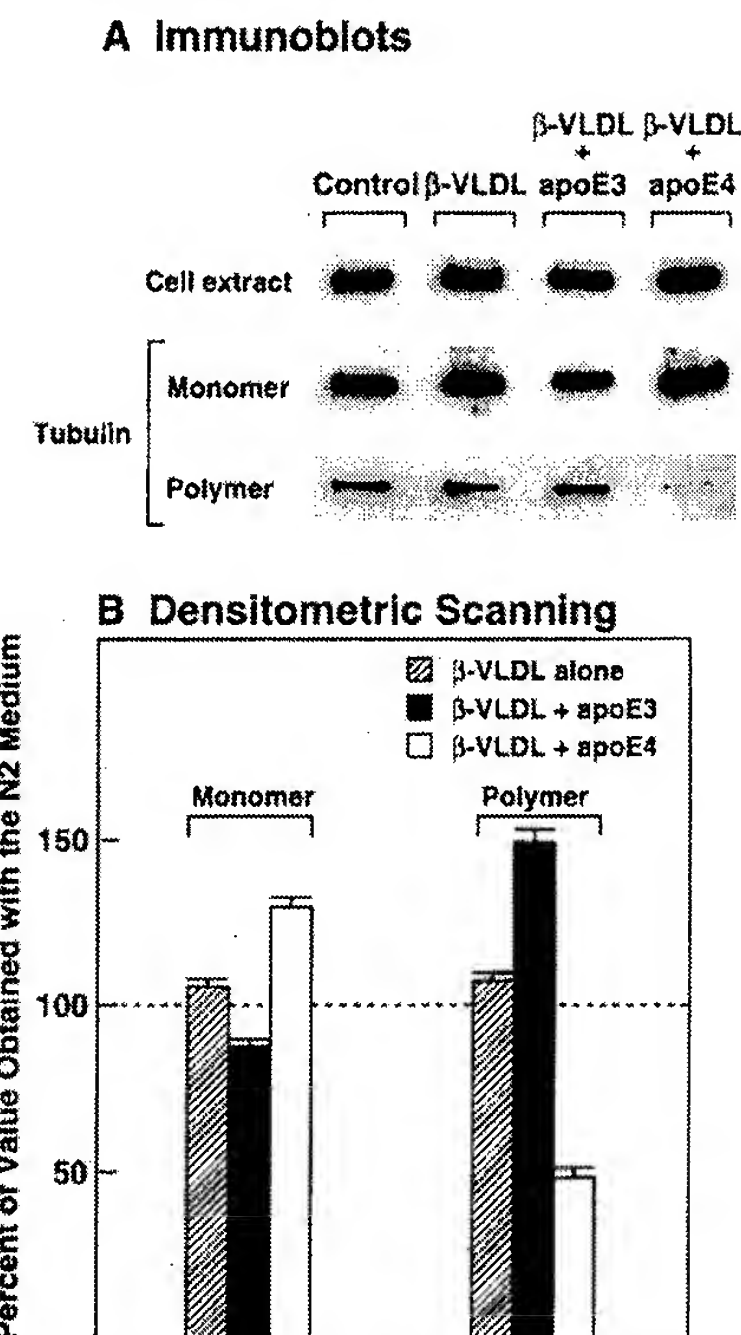


FIG. 10. Immunoblotting of total, polymeric, and monomeric forms of tubulin from Neuro-2a cells. Neuro-2a cells were incubated for 2 days in medium alone (control), in medium containing β-VLDL (40 μg cholesterol/ml), or in medium containing β-VLDL together with 30 μg/ml of either human apoE3 or human apoE4. Following incubation, cell extract was prepared as described under "Experimental Procedures." A, an aliquot of the cell extract containing 50 μg of total protein from each treatment condition was immunoblotted using a monoclonal antibody to α-tubulin. Monomeric and polymeric forms of tubulin were separated from the cell extract by centrifugation, as described under "Experimental Procedures," and immunoblotted for α-tubulin as described above. B, densitometric scanning of immunoblots obtained from three independent experiments performed as described in A. The value for the N2 medium alone was set at 100% (dashed line), and data were calculated as the percent difference between each treatment group and the matched control (N2 medium alone) for each experiment. The percent differences for the different experiments then were averaged. Data are presented as the mean ± S.E.

The mechanism whereby apoE may alter the microtubular system is unclear. However, apoE3 binds to crude microtubule preparations from Neuro-2a cells to a greater extent (2-fold) than does apoE4. These results are consistent with those of Huang *et al.* (44) and Strittmatter *et al.* (45), who demonstrated that apoE3 bound much more avidly to tau and MAP2c, two microtubule-associated proteins, than did apoE4. Tau, MAP2c, or other microtubule-associated proteins may be mediating the binding of apoE to the crude microtubule preparations observed in our studies. In fact, the accumulation and retention of apoE3 in the Neuro-2a cells may reflect an interaction of apoE3 with the microtubules. Based on their biochemical studies, Roses (46) and Strittmatter *et al.* (45) postulated that the interaction of apoE3 with tau might support and stabilize microtubule formation and prevent hyperphosphorylation of tau. Hyperphosphorylated tau is a major component of neurofibrillary tangles, one of the characteristic lesions of Alzheimer's disease, suggesting a role for the microtubular system in the pathogenesis of the disease.

Even though apoE4 does not support neurite outgrowth, it is important to note that apoE4 does not have a general toxic effect on the Neuro-2a cells. Removal of the apoE4 from the cells allows neurite extension to occur. Furthermore, the effect of apoE4 on microtubules does not reflect a general disruption of the cytoskel-

eton. The apoE4 did not affect actin stability, and actin filaments appeared identical in cells incubated with  $\beta$ -VLDL and either apoE3 or apoE4. Furthermore, apoE4 did not effect cell replication, as determined by thymidine incorporation.

In conclusion, these studies suggest that apoE4 might play a role in the pathogenesis of Alzheimer's disease by destabilizing microtubules. In the aging brain, it is known that tubulin concentrations are low, favoring microtubular disassembly (47, 48). In combination with a low tubulin level, the expression of apoE4, which appears to stimulate microtubular depolymerization, may prevent normal neuronal remodeling from occurring later in life, when the disease process occurs. Ongoing studies aimed at elucidating the mechanism responsible for the apoE4-mediated inhibition of neurite extension and the possible involvement of the microtubular system in this inhibition may shed light on the pathogenesis of Alzheimer's disease and other neurodegenerative disorders.

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transradial approach (2 patients) was used to implant stents through 6 or 8 French guiding catheters in 22 vessels (7 LAD, 5 RCA, 5 circumflex, 3 left main, 1 obtuse marginal, 1 SVG); 10 vessels had bifurcations at the site, and 11 had  $> 45^\circ$  curvature. Preprocedure minimum lumen diameter (MLD) was  $1.1 \pm 0.3$  mm, diameter stenosis was  $82 \pm 7\%$ , and lesion length was  $31 \pm 17$  mm (16 to 66 mm). All stents were successfully deployed at the intended site; 4 vessels received multiple GR II stents (maximum 3). The post-stent MLD was  $3.2 \pm 0.5$  mm, with a residual stenosis of  $3 \pm 3\%$ . Post-procedure therapy for 17 patients consisted of ticlopidine for 1 month and aspirin indefinitely; 1 patient with atrial fibrillation received systemic anticoagulation. No subacute thromboses, deaths, myocardial infarctions, or repeat interventions occurred within the first 3 months of clinical follow-up. **Conclusion:** The new features facilitate stent placement compared to the currently available system. Initial clinical results appear promising.

#### 921-43 Late Clinical and Angiographic Outcome of Bailout Coronary Stenting. A Comparison Study Between Gianturco-Roubin and Palmaz-Schatz Stents

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Coronary stenting provides a rapidly applied nonsurgical alternative to correct acute dissections following unsuccessful balloon angioplasty. Although a variety of stents have been shown to be useful in this situation, the influence that the stent design may have in long-term outcome remains uncertain. In the present study, 34 consecutive pts receiving a Gianturco-Roubin (GR) stent were matched to 34 pts receiving a Palmaz-Schatz (PS) stent. Pts were eligible if they had undergone successful coronary stenting for acute dissection following balloon angioplasty and a 6-month control angiographic study had been performed. Group matching was performed according to vessel size (reference diameter [RefD] by quantitative computerized angiography [QCA]), location of target lesion and dissection type (AHA classification). Follow-up clinical data (% free of cardiac death, revascularization and myocardial infarction [Ev-free]) and QCA angiographic follow-up data (late loss and minimal luminal diameter [MLD<sub>fu</sub>], and % of restenosis  $> 50\%$  [Re]) were compared ( $X \pm SD$ ):

	GR	PS	p value
RefD (mm)	$2.9 \pm 0.4$	$2.9 \pm 0.4$	ns
Dissection length (mm)	$10.1 \pm 8.2$	$19.7 \pm 5.7$	ns
MLD post-stent (mm)	$2.4 \pm 0.5$	$2.5 \pm 0.5$	ns
MLD follow-up (mm)	$1.3 \pm 0.9$	$1.8 \pm 0.7$	$< 0.05$
MLD late loss (mm)	$1.1 \pm 0.8$	$0.7 \pm 0.6$	$< 0.05$
Re (%)	15 (44%)	5 (15%)	$< 0.05$
Ev-free (%)	24 (70%)	29 (85%)	$< 0.05$

**Conclusions:** PS stenting for the management of acute coronary dissections after angioplasty provides a better clinical outcome than GR stenting. This benefit is mainly determined by a lower late loss resulting in larger luminal diameter at 6-month follow-up with PS stenting.

#### 921-44 Serial Angiographic Follow-up After Cordis Stent Implantation. — Comparison With Palmaz-Schatz Stent —

Naoya Hamasaki, Hideyuki Nosaka, Takeshi Kimura, Masakiyo Nobuyoshi. *Kitakyushu, Kokura Memorial Hospital, Japan*

The purpose of the present study was to evaluate progress of restenosis after successful Cordis (CD) stent placement and to compare it with Palmaz-Schatz (PS) stent placement by matched lesion analysis. From Jan. '94 to Feb. '95, CD stent placement was attempted in 92 consecutive patients (pts) with 94 lesions (ls) and successful single stent placement was achieved in 65 pts. with 68 ls. Prospective serial angiographic follow-up (F/U) was scheduled for next day, 1 month, 3 and 6 months after procedure. F/U angiogram was completed in 60 pts. with 61 ls. Matching process was based on stenosis location, reference diameter ( $\leq 0.3$  mm) and minimal lumen diameter (MLD  $\leq 0.1$  mm). All 61 ls. were matched identically selected from 586 consecutive series of successful P/S stent placement (Feb. '90–June '94).

	CD	PS	
Reference diameter (mm)	$3.14 \pm 0.44$	$3.14 \pm 0.44$	NS
MLD pre (mm)	$1.04 \pm 0.29$	$0.99 \pm 0.28$	NS
MLD post (mm)	$2.81 \pm 0.29$	$2.89 \pm 0.35$	NS
MLD F/U (mm)	$1.94 \pm 0.58$	$1.99 \pm 0.66$	NS
Relative gain	$0.57 \pm 0.11$	$0.61 \pm 0.13$	NS
Relative loss	$0.28 \pm 0.19$	$0.29 \pm 0.21$	NS

In conclusion, although there was more complicated and restenotic lesions in CD stent group, narrowing process didn't differ between groups in this relatively a small number of cohort.

#### 922 Angioplasty and Restenosis: Clinical

Monday, March 25, 1996, 3:00 p.m.–5:00 p.m.

Orange County Convention Center, Hall E

Presentation Hour: 4:00 p.m.–5:00 p.m.

#### 922-45 Mechanism of Restenosis Following Stenting of Renal Arteries With Nonarticulated Palmaz™ Stents

Sandeep Khosla, Dinesh Shaw, Nancy McCarthy, Tyrone J. Collins, J. Stephen Jenkins, Christopher J. White, Stephen R. Ramee. *Ochsner Clinic, New Orleans LA*

To investigate the mechanism of restenosis following renal artery stenting, computer-assisted quantitative angiographic measurements were made at the proximal, middle and distal segments of the stent in 57 renal arteries treated with nonarticulated Palmaz™ stents in 41 patients (28 male, 13 female, mean age 66 years; range 37 to 84 years). Identical measurements were repeated at 6 month angiographic follow-up (mean  $255 \pm 240$  days). Mean reference vessel diameter (RVD) at baseline, post stent and at 6 months was  $5.1 \pm 1.2$ ,  $5.1 \pm 1.2$  and  $4.9 \pm 1.2$  mm respectively ( $p = NS$ ). Mean minimum luminal diameter (MLD) at baseline, post stenting and at 6 months was  $1.2 \pm 0.6$ ,  $4.9 \pm 1.1$  and  $3.3 \pm 1.2$  mm respectively ( $p < 0.0001$  versus baseline).

	Prox. (mm)	Middle (mm)	Distal (mm)
MLD post-stenting	$4.7 \pm 0.9$	$4.9 \pm 1.0$	$4.7 \pm 0.9$
MLD at 6 months	$3.6 \pm 1.0^*$	$3.7 \pm 1.1^*$	$3.7 \pm 1.1^*$
Lumen loss at 6 months	$1.2 \pm 0.7$	$1.2 \pm 1.0$	$1.2 \pm 0.9$
Stent diameter post-stenting	$5.4 \pm 0.7$	$5.3 \pm 0.7$	$5.3 \pm 0.7$
Stent diameter at 6 months	$4.9 \pm 0.7^\dagger$	$4.8 \pm 0.6^\dagger$	$4.8 \pm 0.7^\dagger$
Stent recoil at 6 months	$0.5 \pm 0.4$	$0.4 \pm 0.3$	$0.3 \pm 0.4$
Stent recoil index <sup>‡</sup>	41%	33%	25%

<sup>‡</sup>Stent recoil index = (stent recoil at 6 months  $\div$  lumen loss at 6 months). \* $p < 0.001$  versus MLD post-stenting. <sup>†</sup> $p < 0.001$  versus Stent diameter post-stenting

Stent diameter at 6 month follow-up was smaller than immediately post-stenting suggesting that restenosis is due to a combination of stent recoil and tissue ingrowth in these patients. **Conclusion:** Late lumen loss following stent deployment in the renal arteries is predominantly due to tissue ingrowth. There is, however, significant stent recoil at 6 months which also contributes to the late lumen loss.

#### 922-46 Apolipoprotein E Isoform and Lipoprotein(a) as Risk Factors for Restenosis After Coronary Angioplasty

Hideya Yamamoto, Michinori Imazu, Takashi Yamabe, Takehiko Watanabe, Hironori Ueda, Koichi Ono, Futoshi Tadehara, Michio Yamakido, Yasuhiko Hayashi. *Hiroshima University, Hiroshima, Japan*

We previously reported serum level of lipoprotein(a) [Lp(a)] was a risk factor for restenosis. To evaluate that apolipoprotein E (apoE) isoform which predisposes to premature coronary artery disease also has any predictive value for restenosis, 103 patients (age;  $63.3 \pm 9.7$ ) who underwent elective PTCA were available for analysis. The quantitative analysis of the stenotic coronary segments was carried out. Restenosis was defined as the presence of  $> 50\%$  stenosis at follow-up. Lp(a) was measured before PTCA. ApoE phenotypes were determined by isoelectric focusing and classified into three categories; E2 (E2/2, E2/3), E3 (E3/3), E4 (E4/4, E4/3). **Results:** The median level of serum Lp(a) was 25.7 mg/dl in patients with restenosis ( $N = 50$ ) compared with 21.1 mg/dl in those without restenosis ( $N = 53$ ) ( $p < 0.05$ ). The restenosis rate was significantly higher in patients with Lp(a) levels  $\geq 30$  mg/dl ( $N = 32$ ) than in those with Lp(a) levels  $< 30$  mg/dl ( $N = 71$ ) (66% vs. 39%,  $p < 0.02$ ). Serum lipids and incidence of restenosis in patients with three apo E were as follows. One subject of E2/4 was excluded.

	E2 (N = 5)	E3 (N = 81)	E4 (N = 16)	
T. chol. (mg/dl)	184.6	208.7	217.3	ns
Lp(a) (mg/dl)	22.8	24.2	17.8	ns
Restenosis	1 (20%)	39 (46%)	11 (67%)	ns
Lp(a) $< 30$ mg/dl (N = 70)	(N = 5)	(N = 54)	(N = 11)	
Restenosis	1 (20%)	18 (33%)	9 (82%)	$p < 0.02$
Lp(a) $\geq 30$ mg/dl (N = 32)	(N = 0)	(N = 27)	(N = 5)	
Restenosis		18 (71%)	2 (40%)	ns

Multiple regression analysis adjusted for age, gender, and total cholesterol revealed that Lp(a) and apoE4 were related with restenosis. Lp(a) (10 mg/dl) (1.52; 1.15–2.10,  $p < 0.01$ ), apoE4 (1.84; 1.04–3.82,  $p < 0.05$ ), (Odd ratio; 95% confidence interval, p value). **Conclusions:** Elevated levels of serum



# Mechanisms of Restenosis

Ward Casscells, MD  
David Engler, PhD  
James T. Willerson, MD

*Restenosis after percutaneous transluminal coronary angioplasty remains a problem, which suggests that we still do not fully understand its mechanisms. We review here the current understanding of the cell biology of restenosis, including clinical correlation (risk factors), randomized clinical trials, human histology, animal models, and in vitro studies. (Texas Heart Institute Journal 1994;21:68-77)*

**R**estenosis after percutaneous transluminal coronary angioplasty (PTCA) continues to be a frustrating problem. Even though it does not confer an increased mortality (because patients with restenosis develop angina rather than myocardial infarction or sudden death), restenosis leads to repeat PTCA or coronary artery bypass grafting, and so increases morbidity and costs.<sup>1</sup> The failure of so many therapies to significantly lower restenosis rates—whether defined as % diameter stenosis, minimum lumen diameter, or late loss—suggests that the mechanisms of restenosis are not well understood.<sup>2,3</sup> This article reviews what is known about these mechanisms. The information about restenosis comes from the following 5 categories: clinical correlation (risk factors), randomized clinical trials, human histology, animal models, and in vitro studies, as discussed below.

## Risk Factors

Few risk factors for restenosis have stood the test of time, and their predictive power is weak—even that of such factors as lipoprotein (a), low high-density lipoprotein (HDL), prior restenosis, total occlusion, diabetes, or location of the left anterior descending coronary artery.<sup>1-9</sup>

Most risk factors related to PTCA are, likewise, weak predictors. However, a patient with angina, residual stenosis, or a positive perfusion scan soon after PTCA is at increased risk. Does this increase occur because a vessel that is already narrow can't afford to lose much lumen, or is it due to the increased thrombotic risk of the high shear at the stenosis and the high turbulence just distal to the stenosis? As Baim and colleagues have pointed out,<sup>4</sup> restenosis may still occur after successful PTCA, because the late loss is to some extent proportional to the initial gain, suggesting that restenosis is proportional to the degree of injury. Better lesion-specific predictors are needed. One leading candidate is cyclic flow variation, which is caused by transient episodes of platelet aggregation and vasoconstriction, and can now be detected in patients by use of a Doppler flow wire.<sup>10</sup>

## Clinical Trials

What mechanistic insights have clinical trials provided? As recently reported,<sup>11</sup> stents may be beneficial because a larger lumen better accommodates a given amount of neointima; but stents may also help by improving flow, resisting spasm, and preventing dissection.

Randomized trials of drug and diet therapies have been disappointing thus far. However, many of the trials have used low doses or too few patients, particularly in the case of heparin,<sup>12</sup> angiotensin-converting enzyme inhibitors,<sup>13</sup> and vitamin E.<sup>14</sup> Thrombosis, angiotensin II, and oxidation may very well play a role in restenosis. Indeed, the recent EPIC trial of an antibody against the platelet IIb/IIIa receptor has found not only a lower rate of early reocclusion after PTCA, but also a decrease in late ischemic events, suggesting inhibition of restenosis.\*

\*Topol E. Personal communication, January 1994.

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**Key words:** Angioplasty, transluminal, percutaneous coronary; cell cycle; constriction, pathologic/recurrence; endothelium, vascular; extracellular matrix; growth factors; muscle, smooth, vascular; risk factors; signal transduction; thrombosis

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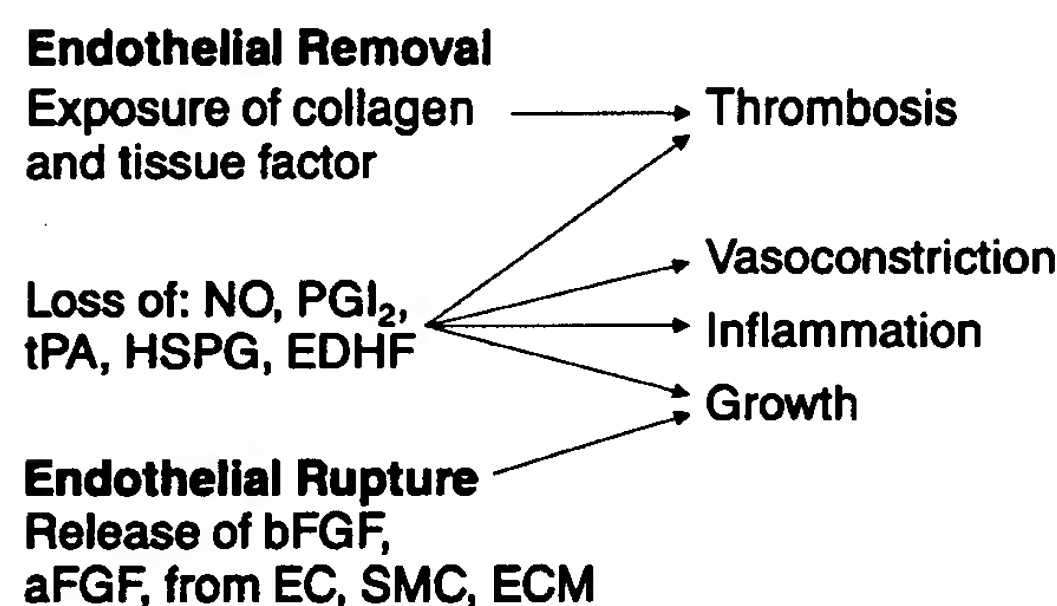
## Time Course of Vascular Balloon Injury

The time course of restenosis can be inferred from data on injury of the rat carotid arteries, from other animal models, and from some autopsy studies.<sup>15-22</sup> Endothelial removal and endothelial death, as well as some smooth muscle death, smooth muscle separation, and smooth muscle stretch, occur within seconds of injury. Over the course of several minutes there is platelet attachment, release, aggregation, and coagulation. During the 1st few days, endothelial and smooth cells and macrophages proliferate and migrate. Over several weeks, synthesis, maturation, and contraction of the extracellular matrix take place, in addition to a process of remodeling in which the whole vessel may get larger or smaller.

## Mechanisms: Current Consensus

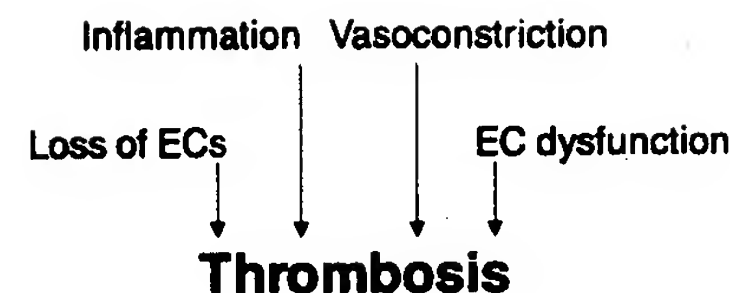
Endothelial removal exposes collagen and tissue factor, leading to thrombosis and to loss of nitric oxide,<sup>23,24</sup> prostacyclin,<sup>25</sup> tissue plasminogen activator (tPA),<sup>26</sup> heparan sulfate proteoglycans (HSPG),<sup>27,28</sup> and endothelial-derived hyperpolarizing factor (EDHF).<sup>29</sup> These losses may result in thrombosis, vasoconstriction, inflammation, and growth. Endothelial rupture causes release of fibroblast growth factors from the cells and their extracellular matrix (Fig. 1).<sup>30</sup>

Thrombosis is a function of inflammation, vasoconstriction, loss of endothelial cells, and endothelial cell dysfunction.<sup>31-34</sup> In this process, platelets release a variety of prothrombotic, vasoconstrictive, and growth-promoting mediators. Some enzymes of the coagulation cascade are also mitogenic for smooth muscle cells (Fig. 2).<sup>35</sup>



**Fig. 1** Loss of endothelium and damage to endothelium promote thrombosis, vasoconstriction, inflammation, and growth through multiple mechanisms.

aFGF = acidic fibroblast growth factor (FGF-1); bFGF = basic fibroblast growth factor (FGF-2); EC = endothelial cells; ECM = extracellular matrix; EDHF = endothelial-derived hyperpolarizing factor; HSPG = heparan sulfate proteoglycans; NO = nitric oxide; PGI<sub>2</sub> = prostacyclin; SMC = smooth muscle cells; tPA = tissue plasminogen activator



- |                    |                       |              |
|--------------------|-----------------------|--------------|
| (Platelet release  | and                   | coagulation) |
| • TXA <sub>2</sub> | • Fn                  | • Xa         |
| • PDGF-AB, BB      | • heparanase          | • thrombin   |
| • 5HT              | • osteonectin (SPARC) |              |
| • ADP              | • LTB <sub>4</sub>    |              |
| • TGFβ1            | • 12-HETE             |              |
| • Tsp              |                       |              |
| • PF4              |                       |              |
| • EGF              |                       |              |

**Fig. 2** Mechanisms of thrombosis in arterial injury.

ADP = adenosine diphosphate; EC = endothelial cell; EGF = epidermal growth factor; Fn = fibronectin; 12-HETE = 12-hydroxyeicosatetraenoic acid; 5HT = serotonin; LTB<sub>4</sub> = leukotriene B<sub>4</sub>; PDGF = platelet-derived growth factor; PF4 = platelet factor 4; TGFβ1 = transforming growth factor β1; Tsp = thrombospondin; TXA<sub>2</sub> = thromboxane A<sub>2</sub>; Xa = activated factor X

Stretch of the smooth muscle cells activates ion channels, which directly promotes smooth muscle cell activation and growth,<sup>36</sup> as does rupture of the internal elastic membrane.<sup>37</sup> Separation of cells exposes them to plasma and platelet mitogens, and disrupts contact inhibition.

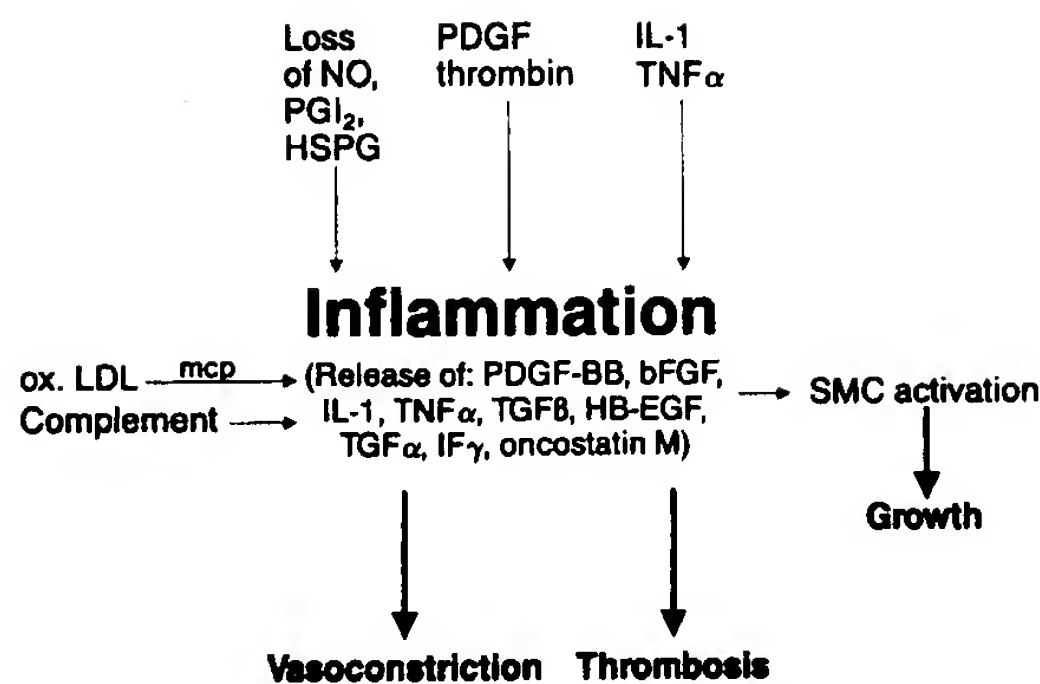
Inflammation is caused by chemoattractants, loss of endothelial anti-inflammatory factors, and activation of specific eicosanoids, adhesion molecules, and cytokines. Macrophages release numerous factors that promote growth, thrombosis, and vasoconstriction (Fig. 3).<sup>38-40</sup>

Endothelial dysfunction is caused by stretch, thrombosis, and inflammation, and persists during endothelial regeneration.<sup>13,40,41</sup> Dysfunctional endothelial cells overexpress plasminogen activator inhibitor-1 (PAI-1), fibronectin, thrombospondin, integrins, selectins, angiotensinogen, angiotensin-converting enzyme, endothelin, and several growth factors, leading to smooth muscle cell activation and growth (Fig. 4).<sup>13,42-44</sup>

In summary, vascular smooth muscle cell activation and growth after balloon injury are now thought to involve multiple mechanisms (Fig. 5).<sup>36</sup>

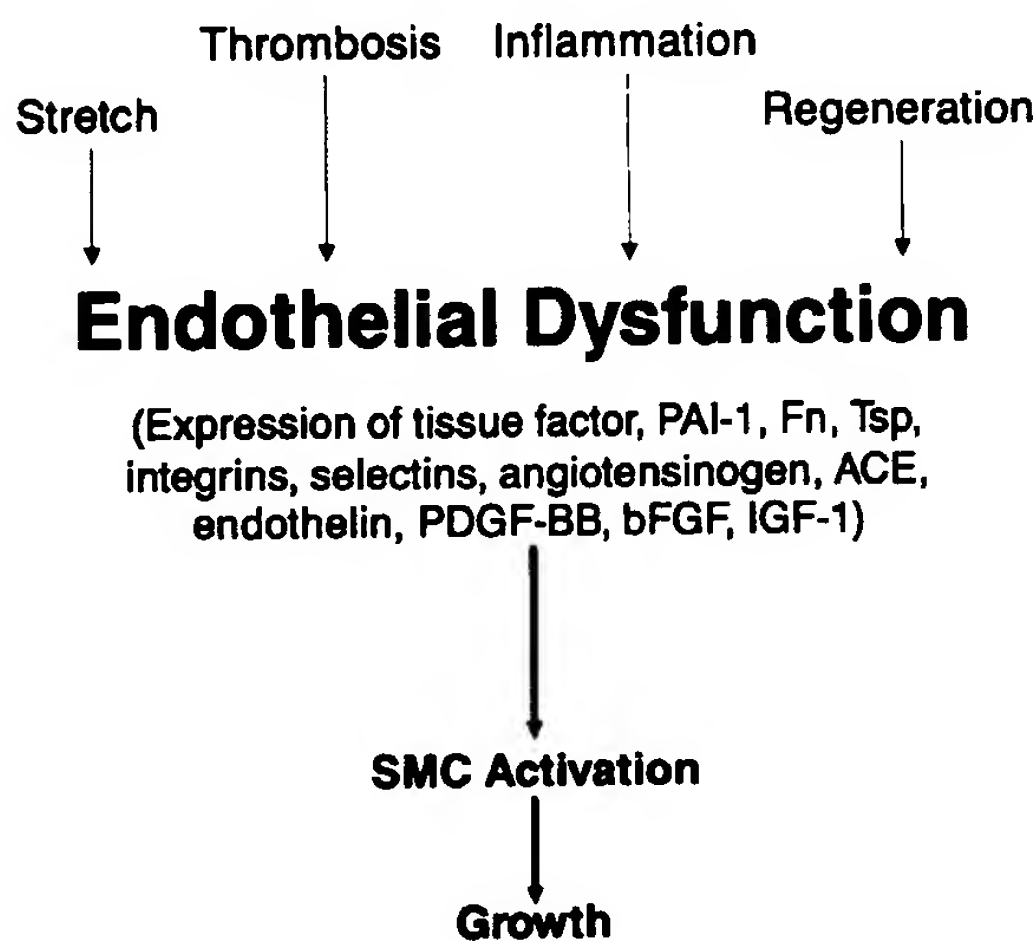
## Recent Trends in Restenosis Research

Poor predictive power of risk factors and disappointing results of clinical trials have led to the reevaluation of several assumptions about restenosis. As a result of this new approach, O'Brien and colleagues have found that there is a rather low rate of smooth muscle cell proliferation in specimens of human



**Fig. 3** Mechanisms and consequences of inflammation after arterial injury.

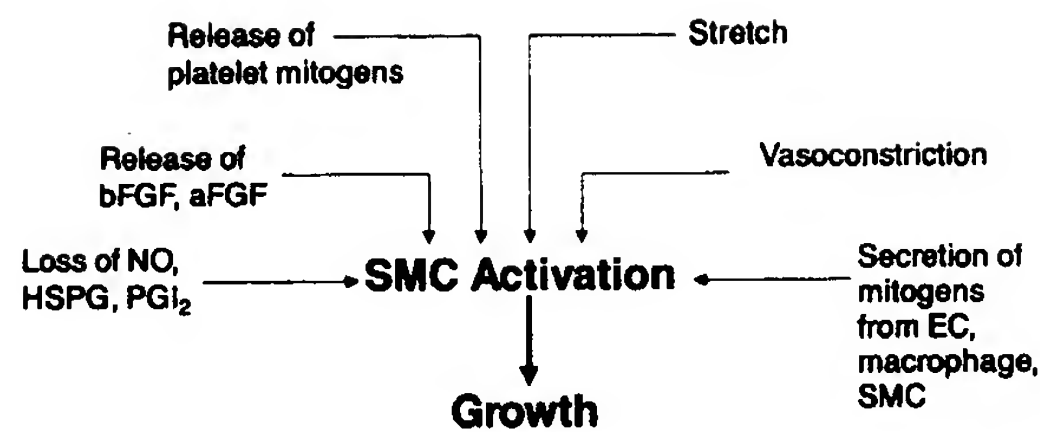
bFGF = basic fibroblast growth factor; HB-EGF = heparin-binding epidermal growth factor; HSPG = heparan sulfate proteoglycans; IF $\gamma$  = interferon  $\gamma$ ; IL-1 = interleukin-1; mcp = monocyte chemoattractant peptide; NO = nitric oxide; ox.LDL = oxidized low-density lipoprotein; PDGF = platelet derived growth factor; PGI<sub>2</sub> = prostacyclin; SMC = smooth muscle cells; TGF $\alpha$  = transforming growth factor  $\alpha$ ; TGF $\beta$  = transforming growth factor  $\beta$ ; TNF $\alpha$  = tumor necrosis factor  $\alpha$



**Fig. 4** Causes and consequences of endothelial dysfunction after arterial balloon injury.

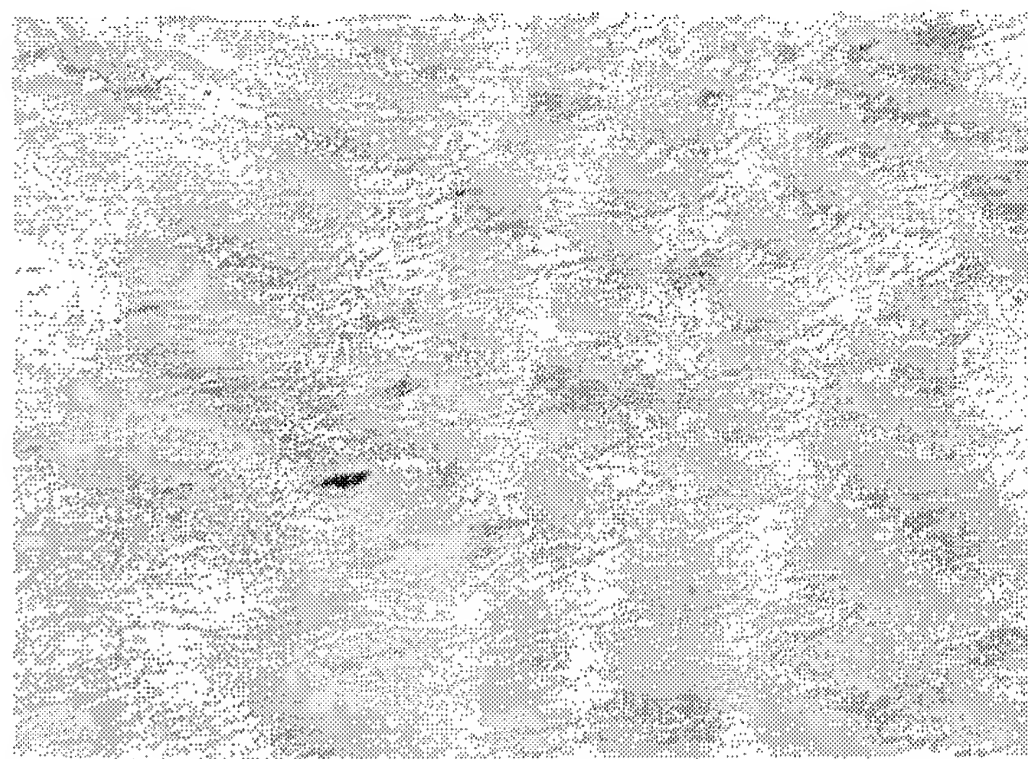
ACE = angiotensin-converting enzyme; bFGF = basic fibroblast growth factor; Fn = fibronectin; IGF-1 = insulin-like growth factor-1; PAI-1 = plasminogen activator inhibitor-1; PDGF = platelet-derived growth factor; SMC = smooth muscle cell; Tsp = thrombospondin

restenosis.<sup>45,46</sup> Moreover, the amount of lumen that is lost cannot be explained by the amount of neointima that develops. This was the subject of several abstracts at a recent American Heart Association meeting in November 1993.<sup>47,48</sup> For example, of the 120 specimens of restenosis we have examined, the specimen in Figure 6 is typical: it shows only a few



**Fig. 5** Multiple mechanisms contribute to smooth muscle cell activation and growth.

aFGF = acidic fibroblast growth factor; bFGF = basic fibroblast growth factor; EC = endothelial cells; HSPG = heparan sulfate proteoglycans; NO = nitric oxide; PGI<sub>2</sub> = prostacyclin; SMC = smooth muscle cells



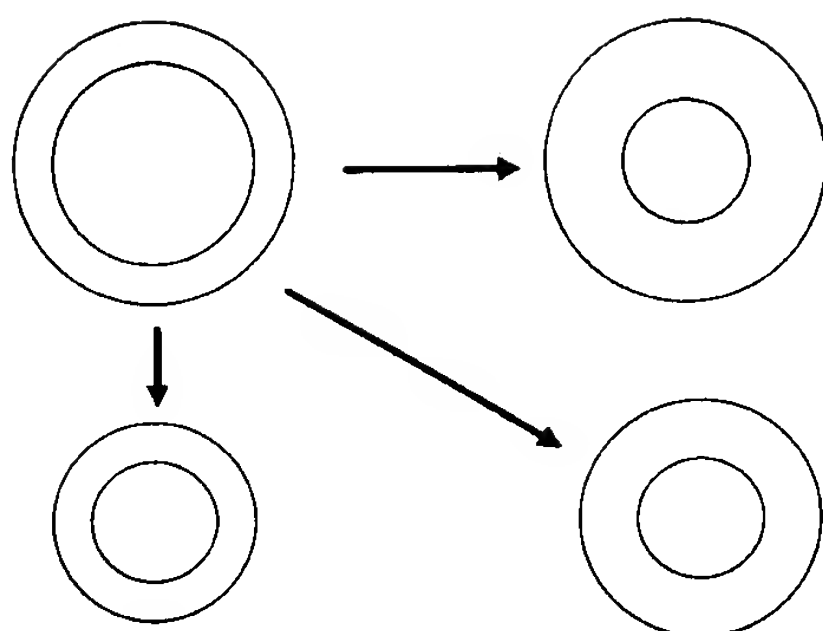
**Fig. 6** Photomicrograph demonstrating the relative paucity of cells (light purple) and the predominance of collagenous extracellular matrix (beige) in a typical specimen of restenosis obtained by directional atherectomy. Shown in dark purple are 3 cells that immunoreact with an antibody to proliferating cell nuclear antigen (PCNA). Some specimens reveal a higher density of cells and PCNA label, particularly when obtained from patients with unstable angina or recent percutaneous transluminal coronary angioplasty.<sup>46</sup>

smooth muscle cells in a sea of extracellular matrix, and very few of these cells show immunoreactivity for proliferating cell nuclear antigen (PCNA). In short, most specimens have a low rate of smooth muscle proliferation, at least at the time that atherectomy is performed for restenosis.

**Remodeling.** Figure 7 depicts 3 possible schemata for the loss of lumen. Recent data<sup>47,48</sup> suggest that the loss of lumen is a function of the creation of a neointima, as well as scar contraction and remodeling. Clearly it is crucial to measure the lumen area and not just to quantify the neointima.

The extracellular matrix constitutes 90% of the neointima. Moreover, the matrix regulates cell migration, proliferation, and matrix production by means of cytoskeletal signals and by posttranslational control of growth factors, receptors, lipids, and nitric





**Fig. 7** Schematic drawing showing the contribution of remodeling and neointimal proliferation to the loss of lumen in restenosis. The diagram at the upper left shows a vessel after successful angioplasty; the upper right shows the traditional concept that the loss of lumen is due to thickening of the vessel wall. The lower left illustrates the view that the loss of lumen is due solely to remodeling, resulting in a smaller vessel. The lower right illustrates the consensus view that remodeling and neointimal development both contribute to the loss of lumen.

oxide.<sup>49-52</sup> Over several weeks, proteoglycans give way to collagens that are contracted by the smooth muscle cells. Little is known about factors that promote extracellular matrix formation and scarring, but transforming growth factor  $\beta$  (TGF $\beta$ ), platelet-derived growth factor (PDGF), vitamin C, and tissue inhibitors of metalloproteases appear to be involved.<sup>53</sup> Factors that may inhibit scarring are plasminogen activators,<sup>54,55</sup> heparin,<sup>56,57</sup> and collagenases.<sup>58</sup>

Recent studies indicate the need to place more emphasis on remodeling of the vessel—an endothelial-dependent process<sup>59,60</sup>—and on the role of the matrix in scar contraction. The data also suggest that there should be less emphasis on the rat carotid artery model, which features a high rate of cell proliferation, and more emphasis on larger animal models. However, the development of transgenic mice with atherosclerotic-like lesions,<sup>61</sup> and of a technique for balloon injury in these animals,<sup>62</sup> may make way for more relevant rodent models.

**Endothelial Regeneration.** There is increased interest in factors that enhance endothelial regeneration, such as fibroblast growth factors (FGFs)<sup>63</sup> and vascular endothelial growth factor (VEGF),<sup>64,65</sup> which acts only on endothelial cells (Fig. 8); less is known about the other factors. A few of the inhibitors of endothelial cells are also being investigated, with particular focus on the effects of oxidized low-density lipoprotein,<sup>66,67</sup> TGF $\beta$ ,<sup>68,69</sup> and cytokines such as interferon- $\gamma$  and interleukin-1.<sup>70-72</sup>

**Smooth Muscle Cell Proliferation and Migration.** Despite the deemphasis on smooth muscle proliferation, it is important to recall that the smooth muscle cells secrete and contract the matrix. The factors that

### Factors that enhance EC

- FGFs
- VEGF
- PD-ECGF
- EGF
- GMCSF
- GCSF
- IL-4
- PGI<sub>2</sub>?
- (Thrombin)
- (HSPG)
- (tPA)
- (Fn)
- (ado)

### Factors that inhibit EC

- Ln
- ox LDL
- TGF $\beta$
- TNF $\alpha$
- IF $\gamma$
- high [Fn]
- (PDGF)
- (NE)
- (5HT)
- (AT II)
- IL-1

**Fig. 8** Factors that enhance or inhibit endothelial cell proliferation and/or migration in vitro and in some cases in vivo. Factors whose actions are variable, depending on culture conditions, are shown in parentheses (for details see references 63-72).

ado = adenosine; AT II = angiotensin II; EC = endothelial cells; EGF = epidermal growth factor; FGFs = fibroblast growth factors; Fn = fibronectin; GCSF = granulocyte colony-stimulating factor; GMCSF = granulocyte macrophage colony-stimulating factor; HSPG = heparan sulfate proteoglycans; 5HT = serotonin; IF $\gamma$  = interferon  $\gamma$ ; IL-1 = interleukin-1; IL-4 = interleukin-4; Ln = laminin; NE = norepinephrine; ox LDL = oxidized low-density lipoprotein; PD-ECGF = platelet-derived endothelial cell growth factor; PDGF = platelet-derived growth factor; PGI<sub>2</sub> = prostacyclin; TGF $\beta$  = transforming growth factor  $\beta$ ; TNF $\alpha$  = tissue necrosis factor  $\alpha$ ; tPA = tissue plasminogen activator; VEGF = vascular endothelial growth factor

promote in vitro smooth muscle proliferation or migration (or both) are shown in Figure 9.<sup>13,15,16,73-76</sup> Although not all of these factors are activated at all times after balloon injury, there is substantial redundancy. Moreover, multiple factors induce each other; for example, FGF is induced by thromboxane A<sub>2</sub>,<sup>77</sup> thrombin,<sup>78</sup> serotonin,<sup>79</sup> and angiotensin II.<sup>80</sup>

Substantial redundancy exists in embryonic development. A number of "knock-out" genes were thought to be critical for the growth and development which, surprisingly, resulted in mice with apparently normal hearts. Examples include the gene for endothelin 1, brain nitric oxide synthase, TGF $\beta$ 1, tenascin, tPA, urokinase plasminogen activator, and PAI-1.<sup>81</sup> However, it is not certain that these mice would respond normally to balloon injury. Moreover, it is unlikely that redundancy is as extensive in the adult as in the embryo. In adult rats, factors such as basic FGF have been neutralized (with antibodies or antisense oligonucleotides), yielding at least transient effects on restenosis.<sup>82,83</sup>

Many factors are known to inhibit endothelial cells, but only a few inhibit smooth muscle cells: bradykinin,<sup>84</sup> atrial natriuretic factor,<sup>85</sup> and prostacy-

\*Dixon R. Personal communication, November 1993.

clin. Other factors such as TGF $\beta$  can inhibit smooth muscle cells, depending on context or on the smooth muscle cell subtype (smooth muscle cells are known to be heterogeneous).

The recent realization that many agonists perform multiple actions provides further evidence of their complexity. For example, thrombin promotes not

just cleavage of fibrinogen but also smooth muscle growth and monocyte chemotaxis;<sup>35</sup> tPA has pronounced effects on cell proliferation, migration, and matrix degradation;<sup>55</sup> and FGF and PDGF act as vasodilators.<sup>86,87</sup>

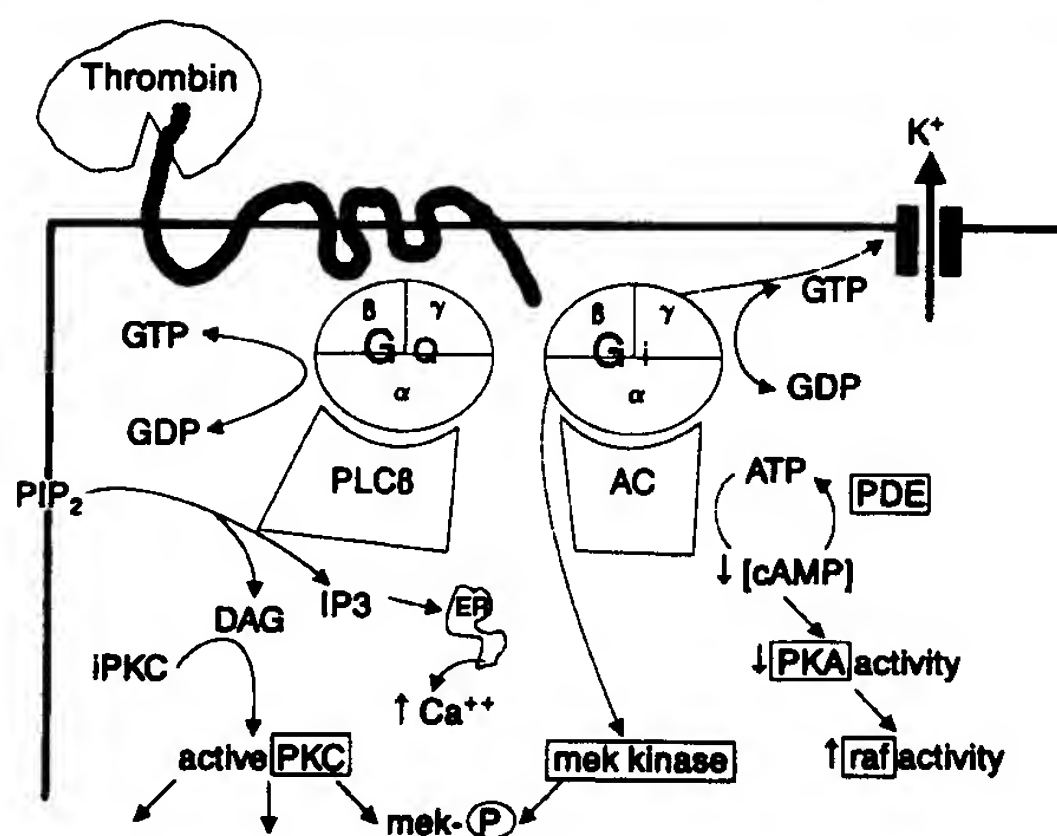
**Receptor Complexity.** It has recently been shown that receptors are much more complex than previ-

Plasma	Thrombus	EC, SMC, MØ
• LDL, HDL	• PDGFs	• FGFs
• 5 HT	• 5 HT	• PDGFs
• NE	• osteonectin (SPARC)	• TGF $\alpha$
• EGF	• PF 4	• EGF
• IGF-1	• Tsp	• IGF-1
• transferrin	• TXA <sub>2</sub>	• Angiotensinogen, ACE
• A II	• 12-HETE	• Tsp
• Ip(a)	• ADP	• Fibronectins
• VIP	• (TGF- $\beta$ )	• HB-EGF
	• thrombin	• IL-1
	• Xa	• (TGF- $\beta$ s)
	• fibrin	• (endothelin)
	• fibronectin	• (PAI-1)
	• (plasmin)	• (IF- $\gamma$ )

**Fig. 9** Factors that enhance proliferation and/or migration of cultured smooth muscle cells are listed beneath their sources.

Those in parentheses can enhance or inhibit depending on context. See text for reviews with detailed references.

AT II = angiotensin II; ACE = angiotensin-converting enzyme; ADP = adenosine diphosphate; EC = endothelial cells; EGF = epidermal growth factor; FGFs = fibroblast growth factors; HB-EGF = heparin-binding EGF; HDL = high-density lipoprotein; 12-HETE = 12-hydroxyeicosatetraenoic acid; 5HT = serotonin; IF $\gamma$  = interferon  $\gamma$ ; IGF-1 = insulin-like growth factor-1; IL-1 = interleukin-1; Ip(a) = lipoprotein(a); LDL = low-density lipoprotein; MØ = macrophage; NE = norepinephrine; PAI-1 = plasminogen activator inhibitor-1; PDGFs = platelet-derived growth factors; PF4 = platelet factor 4; SMC = smooth muscle cells; TGF $\alpha$  = transforming growth factor  $\alpha$ ; TGF- $\beta$  = transforming growth factor  $\beta$ ; Tsp = thrombospondin; TXA<sub>2</sub> = thromboxane A<sub>2</sub>; VIP = vasoactive intestinal peptide; Xa = activated factor X



**Fig. 10** Interacting signal pathways of the 7-membrane-spanning receptors. The box represents a cell in whose membrane lie a potassium channel, a phosphorylated sugar (phosphoinositide bis-phosphate [PIP<sub>2</sub>]), and a 7-membrane-spanning receptor. Dozens of receptors with this same general configuration bind such ligands as norepinephrine, serotonin, and angiotensin II. Upon occupancy of the receptor by the ligand (or in the case of the thrombin receptor, by cleavage of the N-terminus of the receptor, which allows the N-terminus to fold back and activate the receptor), the G protein is activated, leading to substitution of GDP by GTP, thereby releasing the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits for diverse functions and simultaneously decreasing the affinity of the receptor for the ligand. The G protein has GTPase activity that terminates the signaling process.

The G proteins come in general classes such as G<sub>s</sub> (stimulatory), G<sub>i</sub> (inhibitory), and G<sub>o</sub> (which activates phospholipase C $\beta$ ). This enzyme then cleaves PIP<sub>2</sub> into diacylglycerol (which activates protein kinase C [PKC]). The other fragment,

inositol triphosphate, causes release of calcium from the endoplasmic reticulum.

Receptors may alternatively activate inhibitory G-protein complexes (and some may even associate with more than 1 type of G protein, as suggested here). In this case, inhibition of adenylate cyclase results in reduced conversion of ATP to cyclic AMP with ongoing conversion of cyclic AMP to ATP by phosphodiesterase. The decline in concentration of cyclic AMP, the allosteric activator of protein kinase A (PKA), results in a decrease in PKA activity. Depending on the cell type, this can result in altered calcium release, altered transcription, decreased phosphorylation of phospholamban, etc. Of particular interest here, however, is the recent finding that PKA stimulates raf activity, and also that G<sub>i</sub> proteins regulate the phosphorylation of mek. Thus, there are several recently discovered interactions between the PKA and PKC pathways (long known to be regulated by vasoactive hormones and neurotransmitters activating G proteins and the phosphoinositide pathway) and the kinase cascade triggered by growth factors and tyrosine kinases.

Also shown is the direct regulation of the potassium channel by the inhibitory G protein. Not shown are the effects of the  $\beta$  and  $\gamma$  subunits, and the desensitization of the receptor by kinases such as  $\beta$ -adrenergic receptor kinase and arrestin. Also omitted are many details of the phosphoinositide and related pathways, such as IP4 and phosphatidylcholine.<sup>101-104</sup>

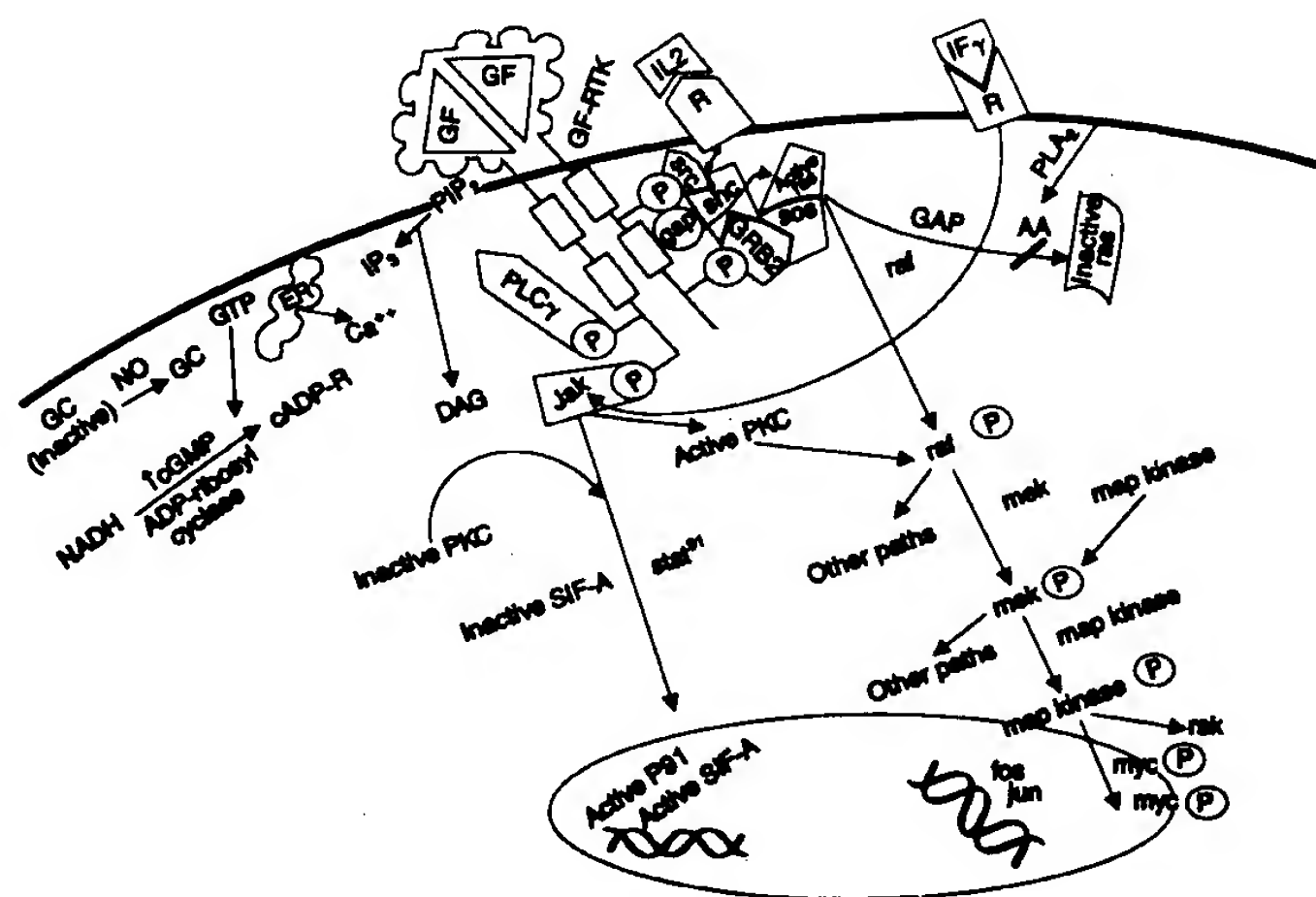
AC = adenylate cyclase; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate; DAG = diacylglycerol; ER = endoplasmic reticulum; GDP = guanosine diphosphate; G<sub>i</sub> = inhibitory G protein; G<sub>o</sub> = activating G protein; GTP = guanosine triphosphate; IP3 = inositol triphosphate; iPKC = inactivated protein kinase C; mek = map kinase kinase; PDE = phosphodiesterase; PIP<sub>2</sub> = phosphoinositide bis-phosphate; PKA = protein kinase A; PKC = protein kinase C; PLC $\beta$  = phospholipase C $\beta$

ously imagined. Williams's group discovered that cells can secrete fragments of FGF receptors which bind FGF.<sup>88</sup> Some cells also seem to produce "dominant-negative" FGF receptors, which are truncated membrane-bound receptors that bind FGFs but cannot phosphorylate intracellular proteins and therefore cannot signal.<sup>89</sup> In addition, FGF receptors have been shown to have alternative tyrosine kinase splicing such that different protein substrates are phosphorylated.<sup>90-92</sup> There are also apparent intracellular receptors for FGF; and dozens of FGF receptor isoforms vary with cell type and cell state.<sup>93-95</sup> To make matters more complicated, FGF receptors (and TGF $\beta$  receptors) have recently been shown to interact with specific components of the extracellular matrix, some of them membrane bound. Finally, there is considerable evidence that basic FGF and its receptors are translocated to the nucleus in specific phases of the cycle of activated cells; however, their exact function is unknown.<sup>96,97</sup>

In the family of 7-membrane-spanning, G-protein-associated receptors, a recent fascinating discovery is that thrombin cleaves its receptor, with the new N-terminus of the receptor then folding back to stimulate the body of the receptor.<sup>35</sup>

The high degree of complexity and redundancy at the level of ligands and their receptors has caused restenosis researchers looking for an Achilles heel to shift their focus to the signal pathways that converge on the nucleus. Berk and others<sup>98,99</sup> have found that inhibition of sodium-hydrogen exchange can inhibit restenosis in animal models. In November 1993, R. Dixon and one of the authors (JTW) reported at the American Heart Association meeting that optimal antisense strategies require that multiple transcripts be attacked simultaneously (such as PDGF and FGF receptors, tenascin, and raf kinase).

Much progress has been made in understanding G proteins, phosphoinositol turnover, calcium release, and protein kinase C activity.<sup>100-104</sup> But in the



**Fig. 11** Generalized pathways for cellular activation (resulting in changes in gene expression and/or mitogenesis) generated from growth factors and cytokines. Growth factor binding to a transmembrane receptor tyrosine kinase (GF-RTK) causes auto- and transphosphorylation of the RTK dimerized molecule (designated as P). Enzymatic cytoplasmic signaling molecules such as src-kinase, phospholipase C- $\gamma$ , Jak-kinase, and GTPase-activating protein (GAP) bind the activated receptor at these sites of phosphorylation directly.<sup>105,106</sup> Other "linker molecules" such as GRB2 also bind activated receptors at these sites of phosphorylation and couple their response to downstream enzymatic signaling molecules such as SOS (other "linker molecules," such as shc, act only to bring signaling molecules closer together for subsequent enzyme-substrate interactions but do not bind activated RTKs directly). Cytokine (interleukin-2 and interferon- $\gamma$ ) binding to cell surface receptors also triggers signaling cascades, using some of the same cytoplasmic signaling molecules as the classical GF-RTKs (such as the src and Jak kinases). These molecules multiply the growth factor and cytokine binding signal many

times over by stimulating an enzymatic phosphorylation cascade of numerous proteins, such as p21<sup>ras</sup> protein (ras); raf kinase (raf); map kinase; map kinase kinase (mek); and ribosomal S6 kinase (rsk); which ultimately act in the nucleus to cause phosphorylation of transcription factors, and subsequent up- or down-regulation of specific gene synthesis, resulting in mitogenesis.<sup>107</sup> More direct routes of gene activation by GF-RTKs are exemplified by phospholipase C- $\gamma$ 's ability to couple the phosphoinositol signaling pathway, in which the phosphatidylinositol biphosphate is cleaved to generate inositol triphosphate, which causes release of free Ca<sup>2+</sup> from the endoplasmic reticulum, and diacylglycerol, which in turn activates protein kinase C. Alternative signaling pathways are exemplified by the ability

of small molecules such as nitric oxide to stimulate guanyl cyclase from the inactive to the active form, causing a rise in the levels of cyclic GMP, and subsequent rise of cyclic ADP-ribose and phospholipase A<sub>2</sub>, to increase arachidonic acid levels and keep ras-GAP in the active state.<sup>108</sup>

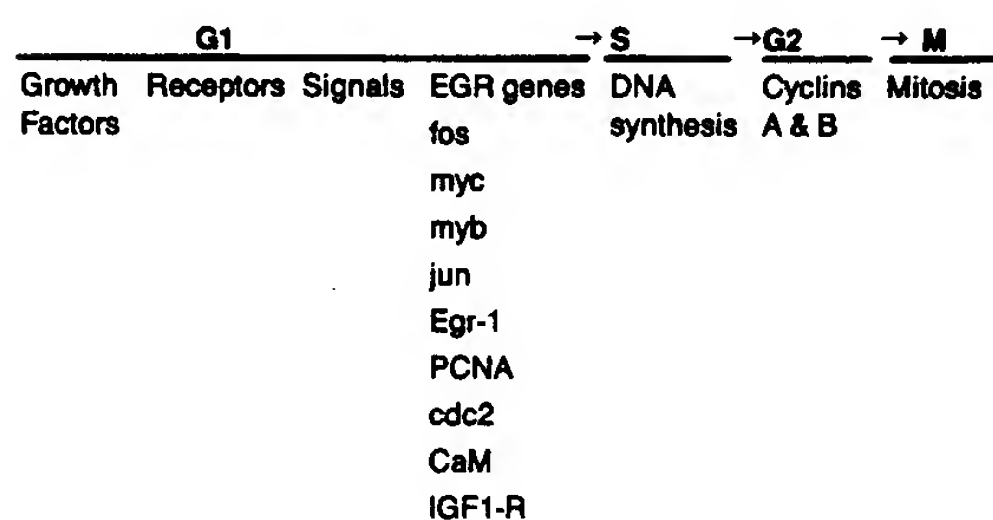
AA = arachidonic acid; ADP = adenosine diphosphate; cADP-R = cyclic adenosine diphosphate-ribose; cGMP = cyclic guanosine monophosphate; DAG = diacylglycerol; ER = endoplasmic reticulum; GAP = GTPase-activating protein; GC = guanylate cyclase; GF = growth factor; GF-RTK = growth factor binding to a transmembrane receptor tyrosine kinase; GTP = guanosine triphosphate; IF $\gamma$  = interferon  $\gamma$ ; IL2 = interleukin-2; IP<sub>3</sub> = inositol triphosphate; mek = map kinase kinase; NADH = reduced form of nicotinamide-adenine dinucleotide; NO = nitric oxide; P = RTK dimerized molecule; PIP<sub>2</sub> = phosphoinositide bis-phosphate; PKC = protein kinase C; PLA<sub>2</sub> = phospholipase A<sub>2</sub>; PLC $\gamma$  = phospholipase C- $\gamma$ ; R = receptor; rsk = ribosomal S6 kinase; SIF-A = sis-inducible factor-A



past few months there have been even more remarkable reports demonstrating interactions of paths previously thought to act independently (Figs. 10 and 11<sup>105-107</sup>; see also reference 108). It has been shown that FGF activates endothelial migration via a different pathway than that used for proliferation. Migration is signaled through a G protein, activation of phospholipase A<sub>2</sub>, 5-lipoxygenase, and ras.<sup>109</sup> Other studies have demonstrated roles for Ca<sup>2+</sup>, cam kinase, and rho. Nitric oxide has recently been shown to utilize a novel mechanism of calcium release by forming cyclic ADP-ribose.<sup>110</sup>

Very recently, yeasts were found to use a 2-component path to the nucleus, featuring phosphorylation on histidine and aspartate.<sup>111</sup> This path most likely functions in smooth muscle cells as well. A new pathway involving a phosphorylated 91-kDa protein has also been discovered.<sup>112-115</sup> As shown in Figure 11, this pathway is distinct from the well-known activation of ras, raf, and map kinases and the subsequent interaction of jun and fos. In the new pathway, a 91-kDa protein is activated and translocated to the nucleus where it participates with activated sis-inducible factor A as a transcription factor.

Figure 12<sup>116-119</sup> shows the cell cycle in a simplified and linearized form. Some of the many early growth response genes are illustrated. Antisense to fos, myc, myb, or PCNA is reported to inhibit smooth muscle proliferation in vitro and, in the case of myb, in vivo. The cyclins are crucial in the final steps of cell division; Dzau's recent work with antisense to cyclin B, PCNA, and cdc2 kinase demonstrates the potential of targeting this final common pathway.<sup>82</sup>



**Fig. 12** The cell cycle of nonmalignant cells is initiated in G1, as shown in this linearized diagram, by synthesis or release of specific growth factors, synthesis of their respective receptors, and transduction of the signals generated by the ligand-occupied receptors as shown in figures 10 and 11. In the nucleus, many early growth response (EGR) genes are expressed, including transcription factors and enzymes, some of which are phosphorylated in a complex pattern leading to DNA synthesis (S), the G2 phase, and then mitosis.<sup>116,117</sup> The tumor-suppressing anti-oncogenes p53 and Rb inhibit the cycle at multiple points (for details see references 118-119).

PCNA = proliferating cell nuclear antigen; EGR = early growth response; IGF1-R = insulin-like growth factor-1 receptor; M = mitosis

In conclusion, it is possible that all the relevant mechanisms have now been identified; if so, study of their regulation, in vivo, should finally provide an understanding of restenosis.

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## Articles

# Polymorphism of the Apolipoprotein E Gene and Early Carotid Atherosclerosis Defined by Ultrasonography in Asymptomatic Adults

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## ► Abstract

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Clinical and autoptical studies have suggested a predisposing role of the allele E4 of apolipoprotein E (apoE) in the development of atherosclerosis and cardiovascular disease. To investigate the possible contribution of apoE allele polymorphism to the carotid intima-media thickness (IMT) as assessed by ultrasound, we studied 260 asymptomatic nondiabetic subjects (121 men, 139 women; mean±SD age, 53±7 years), randomly selected from the population register of the inhabitants of Trieste, Italy. B-mode ultrasound was used to quantify the maximum IMT at 12 sites on the near and far wall of the common, bifurcation, and internal carotid arteries. ApoE genotypes were determined from amplified apoE sequences by restriction isotyping. The frequencies of E2, E3, and E4 alleles were 0.073, 0.827, and 0.100, respectively. As expected, subjects with E4 allele had the highest levels of total serum cholesterol and LDL cholesterol, subjects with E2 allele had the lowest levels, and those with E3 genotype had intermediate levels. The echographic measurements of carotid IMT showed increasing values from E2 to E4 carriers. After adjustment for total and LDL cholesterol serum levels, triglycerides, ratio of LDL to HDL cholesterol, age, sex, and body mass index, ANCOVA showed that the common carotid IMT was significantly greater ( $P=.029$ ) in subjects with E4 allele compared with E3 carriers. Our data confirm the influence of apoE4 on cholesterol levels and clearly show that apoE genotype affects carotid atherosclerosis in its early stages in middle-aged asymptomatic subjects.

**Key Words:** apolipoprotein E • polymorphism • carotid atherosclerosis • ultrasonography

## ► Introduction

Apolipoprotein E is one of the major proteins involved in catabolism of triglyceride-rich lipoproteins (VLDL and remnants). ApoE acts as ligand for two receptors: the "remnants" receptor and the apoB/E receptor. In humans, the gene locus for apoE is polymorphic: three common alleles, E2, E3, and E4, code for three major apoE isoforms in plasma. ApoE3 is the predominant isoform; the other two isoforms differ by an amino acid substitution: apoE4 differs at position 112 (Cys→Arg) and apoE2 at position 158 (Arg→Cys). These substitutions affect ligand binding of triglyceride-rich lipoproteins to the remnants and apoB/E receptors, thus affecting cholesterol serum levels.<sup>1 2</sup> E2 allele is associated with lower LDL-C levels, and E4 allele with higher LDL-C levels, compared with E3 allele.<sup>1 3</sup> Population studies have demonstrated that the different ethnic and geographic distributions of apoE isoforms are associated with a different prevalence of dyslipidemia and CAD.<sup>4 5 6</sup> In Europe, there is a clear-cut gradient for the allele E4 frequency that increases from the south to the north, and it is associated with an increased incidence of CAD.<sup>7</sup>

Clinical and autoptical studies have suggested a predisposing role for E4 and a protective role for E2 in the development of

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atherosclerosis and cardiovascular disease.<sup>8 9 10 11</sup> Up to now, no data are available on the role of apoE isoforms in early asymptomatic carotid atherosclerotic lesions *in vivo*. Atherosclerosis is a disease of the arterial wall, with increasing wall thickness representing an early event in the progression of the disease. In the past decade, ultrasound assessment of the arterial wall allowed *in vivo* study of atherosclerosis even in its early stages.<sup>12 13 14 15 16</sup> The purpose of this study was to investigate the relationship between polymorphism of the apoE gene and carotid IMT as assessed by ultrasound in a group of middle-aged asymptomatic subjects.

## ► Methods

### Subjects

The study subjects were randomly selected from the population register of the inhabitants of Trieste, northeastern Italy. A total of 260 subjects (121 men, 139 women) aged 45 to 65 years (mean±SD, 53±7 years) who were nondiabetic and asymptomatic for cardiovascular disease were invited to participate in the study. To exclude cardiovascular diseases, all subjects underwent a clinical examination and interview that included the Rose Cardiovascular Questionnaire.<sup>17</sup> Diabetic patients were excluded on the basis of a previous history and/or fasting serum glucose levels ≥7.8 mmol/L. Current medications were recorded, and subjects taking lipid-lowering drugs were excluded. The presence or absence of premature CAD among first-degree relatives (parents and siblings) was recorded. A smoking habit was defined as current smoking. Subjects were considered to have hypertension if a history of hypertension requiring treatment was recorded.

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During the screening visit, weight and height were measured with subjects in light clothing without shoes. BMI was calculated as weight divided by height squared. Waist circumference was measured at the level of the umbilicus and hip circumference at the level of the greatest hip girth, with the subject standing and breathing normally. Body fat distribution was measured as waist-to-hip ratio. Informed consent was obtained from each subject. None of the invited subjects refused to participate in the study.

### Lipid Analysis

Venous blood obtained from subjects was collected after an overnight fast in tubes containing EDTA. Total serum cholesterol and triglyceride levels were assayed enzymatically (Boehringer-Biochemia). HDL-C was measured enzymatically after precipitation of apoB containing lipoproteins with Mg-phosphotungstate. LDL-C level was then computed with the Friedewald formula.<sup>18</sup>

### DNA Analysis for ApoE Genotypes

DNA was extracted from the frozen cellular blood component, and apoE genotypes were determined with a modified method as described by Hixson and Vernier.<sup>19</sup> A section of apoE DNA that contains the genotype-differentiating sites was amplified by PCR.



The DNA samples were preheated at 96°C for 5 minutes in a 50-μL PCR reaction buffer including dNTPs and *Taq* polymerase. The preheating was followed by 30 cycles of 96°C for 1 minute, 63°C for 1 minute, and 70°C for 45 seconds. The PCR product (244 bp) was subjected to *Hha* I digestion for 2 hours at 37°C, and the reaction mixture was loaded onto 1% polyacrylamide nondenaturing gel and electrophoresed. The gel was then treated with ethidium bromide, and the DNA fragments were visualized by ultraviolet illumination. The PCR isoform typing results were checked in 10% of the samples by the direct sequencing of amplified DNA.<sup>20</sup> No difference between the enzymatic and DNA sequencing methods was found.

### Ultrasound Assessment of the Carotid Arteries

The ultrasound evaluation of the extracranial carotid arteries was performed using the Biosound 2000 II B-Mode ultrasound system with an 8-MHz probe. The scanning protocol used in this study was detailed elsewhere<sup>21</sup> and is similar to that described by Crouse et al.<sup>22</sup> The protocol entails the longitudinal scanning of the near and far wall of the left and right carotid arteries at the distal common carotid artery, carotid bifurcation, and proximal internal carotid artery. The common carotid was defined as the portion 10 mm below the dilatation of the bulb; the bifurcation was defined proximally by this landmark and distally by the tip of the flow divider; and the internal carotid artery was defined as the portion 10 mm above the tip of the flow divider. In summary, 12 carotid walls in each subject were scanned and analyzed; the measurements were performed three times in real time with an electronic caliper to the nearest 0.1 mm. The mean of these measurements was used in the analysis. Each of the 12 sites was examined with a circumferential scan; of the multiple longitudinal scans, those showing the maximum IMT were selected. An aggregate score was calculated on the basis of the extent of IMT in the 12 carotid walls: the mean maximum IMT represents the mean of the 12 individual maximum thicknesses. All the examinations were performed by two trained physicians certified by the Division of Vascular Ultrasound Research of the Bowman Gray School of Medicine (Wake Forest University, Winston-Salem, NC) during the CAIUS trial.<sup>23</sup> Examinations in which the artery wall was not visualized or showed poor imaging or extensive shadowing were excluded from the statistical analysis.

### Statistical Analysis

Data analysis was performed with BMDP/Dynamic (release 7.0) software. Continuous data were summarized with the mean as a measure of central tendency and standard deviation as a measure of dispersion. The relationship between variables was tested by the least-squares method. Bivariate correlation was tested by the Pearson product-moment correlation coefficient. Group means were compared by one-way ANOVA. A multiple comparison test (Scheffé's method) was used to test the difference between pairs of means. To adjust for the influence of covariates on the outcome variable, ANCOVA was also used. The difference between categorical data tabulated in 2x4 contingency tables was tested by  $\chi^2$  statistic. Because triglyceride concentration and IMT of the arterial wall were not normally distributed, they were logarithmically transformed in statistical analysis. A value of  $P < .05$  (two-sided) was chosen as the limit of statistical significance.

## ► Results

The frequencies of E2, E3, and E4 alleles in the population included in this analysis were 0.073, 0.827, and 0.100, respectively. We regrouped subjects into those with E2 alleles, those with E3/3 genotype, and those with E4 alleles. We excluded 6 subjects (1.9%) with E2/4 genotype because it was difficult to assign them to any of the three groups. Therefore, data analysis was performed on 32 subjects with E2 allele, 177 with E3 genotype, and 45 with E4 allele. As shown in Table 1, the distribution of apoE alleles between men and women was not different. Age was similar in the three groups. There were no significant differences in BMI, waist-to-hip ratio, smoking habit, hypertension, and positive family history of premature CAD between apoE allele groups. The mean values of lipids in the apoE genotypes are reported in Table 2. After adjustment for age, sex, and BMI, levels of total cholesterol, LDL-C, and LDL-C to HDL-C ratio were significantly higher in subjects with E4 alleles than in those with E2 and E3 alleles ( $.005 < P < .05$ ). In the overall sample, IMT at the common carotid and bifurcation arteries was found to be positively correlated with age, sex, LDL-C, and LDL-C to HDL-C ratio ( $.001 < P < .05$ ). IMT at the same sites was inversely correlated to HDL-C ( $P < .02$ ). Table 3 gives the sum of the maximum IMT of the near and far walls of the left and right carotid arteries at the three identified sites. Subjects who were E2 carriers had the lowest carotid IMT at each of the measured sites, whereas the highest values were observed in those with E4 alleles. One-way ANOVA showed that IMT at the common carotid artery was significantly greater in the E4 group than in both E2 and E3 groups ( $P < .05$ ). After controlling for age, sex, BMI, total cholesterol, triglycerides, and LDL-C to HDL-C ratio, IMT at the common carotid artery remained significantly different in the three allele groups ( $P = .029$ ). However, a multiple comparison test showed that the significant F ratio of ANCOVA was due to the difference between the E3 and the E4 groups. After adjustment for age, the estimated percent variance for IMT explained by apoE genotype was approximately 6%. After adjustment for total cholesterol and LDL-C serum levels, fasting triglycerides, and LDL-C to HDL-C ratio, as well as age, apoE genotype still explained approximately 3% of the IMT variance.

**View this table:** **Table 1.** Age, Sex, Anthropometric Data, and Main Cardiovascular Risk Factors According to Apo E  
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**View this table:** **Table 2.** Plasma Lipids According to Apo E Genotypes  
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**View this table:** **Table 3.** Sum of Maximum IMT of Left and Right Carotid Arteries According to Apo E Genotypes  
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## ► Discussion

Because the classic risk factors do not explain all cardiovascular morbidity and mortality, new risk factors have been sought. In recent years, apolipoproteins have been one of the main targets of interest.<sup>24 25 26</sup> ApoE modulates lipoprotein transport and metabolism, and its polymorphism explains about 7% of cholesterol variation at the population level.<sup>1</sup> Accordingly, in our study, E4 allele has been found to be associated with higher cholesterol levels and a higher LDL-C to HDL-C ratio compared with E2 and E3 alleles. However, the key finding in this study is a significant relationship between apoE polymorphism and early carotid atherosclerosis in a sample of a middle-aged population, as indicated by highest values of carotid IMT in E4 carriers. Although many studies have found a higher frequency of the E4 allele in CAD patients than in healthy subjects,<sup>10 11 27 28 29 30</sup> and a more severe atherosclerosis compared with other phenotypes, as far as we

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are aware, a significant association with early carotid atherosclerosis in asymptomatic subjects has not been documented previously. Our results for E4 allele disagree with those of the recent cross-sectional study by de Andrade et al,<sup>31</sup> who found an unexpected association between the E2 allele and carotid artery atherosclerotic disease identified by B-mode ultrasonography. The most likely causes of the discrepancy with our results are the study design and the statistical methods used for data analysis. Furthermore, the 95% confidence intervals for the odds ratios estimated by the authors were rather wide, suggesting that the association between carotid atherosclerosis and different apoE alleles was weak, even for E2/3 allele. Nevertheless, these data raised the question of whether carotid atherosclerosis depends not only on the direct effect of the apoE polymorphism on lipid metabolism and the atherosclerotic process but also on the heterogeneity of the study population with respect to cardiovascular risk factors. In our study, the echographic measurement of carotid IMT showed increasing values from E2 to E4 carriers, and this association was statistically significant at the common carotid artery. In agreement with other studies,<sup>21 32</sup> our asymptomatic subjects showed the greatest IMT at the carotid bifurcation, with increasing values from E2 to E4 carriers; however, the differences among the three allele groups did not reach statistical significance because of the wide IMT range measured at the bifurcation due to ultrasonographic complexity of this area.

These findings are consistent with those of the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Study,<sup>8</sup> in which the extent of atherosclerotic involvement of thoracic and abdominal aorta was assessed in young male subjects who died unexpectedly of unrelated causes. The greatest involvement was in those with the E4 allele and the least in those with the E2 polymorphism; this genotypic effect was independent of cholesterol levels. Considering the fact that apoE polymorphism modulates LDL-C levels and that the prevalence of early carotid atherosclerosis was higher in hypercholesterolemic subjects than in control subjects,<sup>21 33 34 35</sup> it remains questionable as to whether LDL-C levels represent a confounding factor when the risk for carotid atherosclerosis is assessed according to apoE polymorphism. As expected, in the present study, carotid IMT was significantly related to the aging process, LDL-C levels, and LDL-C to HDL-C ratio. Nevertheless, after adjustment for these covariates and the other potential confounding factors, IMT at the common carotid artery remained significantly greater in the E4 allele group than in the E3 genotype group. The explained variance for IMT in the common carotid artery due to apoE genotype was approximately 6% after adjustment for age, quite similar to values for apoE effects on serum cholesterol concentration found at the population level.<sup>1</sup> After adjustment for total serum cholesterol and LDL-C levels, apoE genotype still explained approximately 3% of the variance for IMT in the common carotid artery, consistent with the variance for total lesions in the thoracic and abdominal aorta found by PDAY investigators in young males.<sup>8</sup> However, Bergeron and Havel<sup>36</sup> have recently found that the postprandial increases in triglyceride-rich lipoproteins are prolonged in persons with an apoE4/3 phenotype compared with an apoE 3/3 phenotype. In our subjects, lipid levels were determined after an overnight fast. Therefore, we do not have information on serum levels of chylomicron and VLDL remnants, which are known to be atherogenic<sup>37</sup> and may well account for the 3% variance in carotid IMT that we observed in apoE4 carriers. These data support the view that apoE genotype affects both cholesterol levels and carotid atherosclerosis in its early stages in middle-aged asymptomatic subjects. More information is needed to determine whether apoE genotype affects atherogenesis independently of serum lipid profile.

► **Selected Abbreviations and Acronyms**

apo	=	apolipoprotein
BMI	=	body mass index
CAD	=	coronary artery disease
HDL-C	=	HDL cholesterol
IMT	=	intima-media thickness
LDL-C	=	LDL cholesterol
PCR	=	



polymerase chain reaction

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